1-1-1985

Mechanisms of resistance in corn against maize dwarf mosaic virus.

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MECHANISMS OF RESISTANCE IN CORN AGAINST MAIZE DWARF MOSAIC VIRUS

A Dissertation Presented

By

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Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 1985

Department of Plant Pathology

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This Dissertation is Dedicated to My Wife,

Chen Don

ACKNOWLEDGMENT

I wish to thank a fine teacher and good friend of mine, Dr. George N. Agrios. This work can not be accomplished without his consistent support and instruction. The numerous mind-entertaining discussions between the teacher and the student was the key element of my maturation. Throughout the period of this research, it was the helpful hands of my colleague and fellow graduate students which add on my energy. Also the many suggestions of Dr. M. S. Mount and Dr. G. Hochmuth were of great value to this research. All helps will be thankfully remembered.

The writing of this thesis was done during my postdoctoral fellowship at Rutgers University. Special thanks is given to Dr. T. A. Chen for his generous support and permission in using the computer facilities. I also thank Dr. W. A. Torello for reviewing this dissertation.

ABSTRACT

MECHANISMS OF RESISTANCE IN CORN AGAINST MAIZE DWARF MOSAIC VIRUS

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The mechanisms of resistance in corn against maize dwarf mosaic virus strain B(MDMV-B) were studied by using the corn varieties Pa405 and Bsq (resistant) and Ma5125 (susceptible). MDMV-B replicated to high titer and spread locally in the inoculated leaves, but systemic virus spread occurred only in the susceptible variety. The infectivity of MDMV-B extracted from resistant plants was comparable to that from susceptible plants. When uninoculated leaf tissues of plants previously inoculated with MDMV-B were challenge-inoculated, high titer of MDMV-B was produced in the challenged tissues. Spread of MDMV-B in actinomycin D-treated inoculated plants was not significantly different from that in control plants. A new fluorescent antibody staining technique, which allows precise location of virus infected cells in a leaf area, was developed to investigate the profile of MDMV-B spread in corn leaves. The number of infection loci in mechanically inoculated leaves was higher in Bsq than in Pa405 and Ma5125. The rate of cell-to-cell spread in Pa405 and Bsq was either higher or equal to that

in Ma5125. Six days after inoculation many discrete secondary infection loci developed outside the inoculated area of leaves of Ma5125 but not of Pa405 and Bsq. The MDMV-B-infected area in leaves of Pa405 and Bsq was generally continuous. The rate of long-tern spread of MDMV-B in inoculated leaves was higher in the susceptible than in the resistant varieties. In leaves of susceptible plants the virus spread mostly towards the stem. Failure of virus to travel through the leaf vascular system is proposed as the mechanism of corn resistance to MDMV-B.

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INTRODUCTION

Resistance determined by many genes generally lasts longer and is effective against a wider spectrum of different races of a pathogen than resistance determined by a single gene. Polygenic resistance is, therefore, more desirable as far as disease management is concerned, however, because of the numerous genes involved, it is more difficult to manipulate in a breeding program. In polygenic resistance, expression of an individual gene may be responsible for just one of several steps of disease resistance. Resistance mechanisms that result, for example, in fewer infection sites, reduced virus multiplication, or slower or limited virus translocation are probably controlled by groups of genes. Such groups, however, are made of relatively fewer genes than the genes responsible for the entire process of disease resistance.

Determination of the specific mechanism(s) of resistance can be very helpful in the identification of specific resistance genes. Development of well-defined assays, aiming at pinpointing the specific mechanisms of resistance in the germplasm, help monitor these genes in breeding programs and help clarify the confusion about the genetics

of resistance in plants to many viruses. This study was undertaken in order to obtain information on the apparently polygenic resistance in maize to maize dwarf mosaic virus (MDMV).

MDMV is one of the most important viruses affecting corn in the continental United States and since the mid-1960's has caused widespread economic losses (Gordon, et al., 1981). Since 1965, breeding for resistance against MDMV has become an integral part of corn breeding in several states (Findley, et al., 1981). Many efforts have been made to study the genetics of resistance of corn against MDMV. According to most studies, resistance is probably conditioned by relatively few major genes and a population of minor genes, but no study has demonstrated clearly the exact number or nature of the resistance genes. The difficulties are primarily due to (1) near continuous distribution of symptom expression in segregating generations, (2) whether the number of plants with symptoms increases with time between inoculation and evaluation. Thus there is no suitable criterion which researchers can use to identify specific resistance phenotypes. One way by which these problems can be solved is to develop assays which will determine the specific mechanisms of resistance. Researchers will then be able to precisely measure the effects of individual genes in genetic studies of resis-

tance. In the present work, resistant and susceptible corn varieties of diverse origin were used to investigate the mechanisms of resistance of corn against MDMV-B. We attempted to obtain information on (1) the immunity of corn varieties against MDMV-B, (2) the number of infection loci upon mechanical inoculation with MDMV-B are different in resistant and susceptible varieties, (3) the rate of cell-to-cell spread of MDMV-B in resistant and susceptible varieties, (4) the rate of long-term spread of MDMV-B, and (5) whether uninoculated parts of resistant varieties aquire resistance to infection. In order to follow the profile of spread of MDMV-B a new immunofluorescent staining procedure was developed.

C H A P T E R II

REVIEW OF LITERATURE

Mechanisms of Plant Resistance to Virus

The types of resistance in plants to viruses, as proposed by Russell (1978), were (1) immunity, (2) resistance to infection, (3) resistance to establishment and spread of virus in host plant, (4) resistance to virus multiplication, (5) tolerance, (6) resistance to vectors. Russell's classification is a convenient working frame rather than a rigorous delineation of the types of resistance. One often finds that one type of resistance also contributes to another. Only the first four types of resistance will be discussed because those are of primary concern in the present work.

Immunity

Immunity is a form of protection in which upon inoculation of a plant with virus no symptoms or replication of virus can be detected. We may say that, in general, most viruses are unable to attack most plants. The truth of this becomes evident when we consider the number of diseases of limited host range. It is an exception rather

than the rule to find a disease capable of affecting a high percentage of plant species. The nature of natural immunity is not really known. However, Holmes (1955) proposed that "immunity to specific diseases in nature may constitute random assemblages of similar genetic mechanisms in grouping of a higher order.". As a result, there are too many genes of resistance for the virus to overcome. Holmes further stated that "intentionally assembling minor resistances to a disease eventually would achieve complete hereditary immunity.". This concept of resistance closely meets the characteristics of horizontal resistance described by van der Plank (1968). In the classification of Russell, immunity may well be the combination of all or parts of the other types of resistance.

Resistance to Infection

Resistance to infection, as conceptualized by Russell, is resistance to adsorption or penetration of viruses. The chance of becoming infected is smaller in resistant than in susceptible varieties. Troutman and Fulton (1958) reported that , when tobacco leaves were mechanically inoculated with the necrotic strain of cucumber mosaic virus (CMV), only about one-twentieth as many local lesions developed on leaves of a resistant variety (TI245) as on leaves of a susceptible variety. The resistance of TI245 was corre-

lated with reduced number of ectodesmata in the outer walls of epidermal cells (Thomas and Fulton, 1968). They proposed that fewer infection sites in the resistant variety, as a result of reduced number of ectodesmata, was the mechanism of resistance.

The hypersensitive reaction of White Burley tobacco to tobacco necrosis virus (TNV) was characterized by the development of non-self-limiting necrotic local lesions. Recently, Pennazio et al. (1983) showed that prior TNV inoculation of White Burley tobacco followed by challenge inoculation with the same virus exhibited a systemic aquired resistance against the establishment of challenge-infection sites, but where lesions became established they developed normally. The progressive radial spread of the lesions and the high amount of TNV antigen in the outer halo of the lesions indicated that mechanisms restricting TNV spread and multiplication were not operating. They concluded that the mechanism of acquired resistance in White Burley tobacco was against infection with TNV.

Resistance to Virus Spread in Host Plant

The most striking resistance mechanism affecting the spread of viruses within plants is hypersensitivity which is expressed by rapid death of infected cells. In extreme cases in which only one or two cells are

involved,hypersensitivity resembles immunity since it may not be detected. However, the necrotic response associated with local lesions has often been questioned as a mechanism of restricting spread of viruses. Weintraub and Ragetli (1961) reported that the walls of cells ,in a radius of about 50 cells surrounding the lesions, contain predominantly calcium pectate. Such modification of middle lamella may play a role in restricting the spread of viruses. Shimomura and Dijkstra (1975) noted that callose was detected early during local lesion formation in tobacco mosaic virus inoculated leaves of Nicotiana glutinosa and its characteristic fluorescence grew weaker as tissue browning progressed, usually disappearing entirely when necrosis was completed. They suggest that if callose plays a role in restricting virus spread it must happen at an early stage of the infection, since once the virus invades veins of the inoculated leaves, it spreads rapidly through the entire vascular system. When Samsum NN tobacco was infected by tomato spotted wilt virus (TSWV) and tomato black ring virus (TBRV), callose was deposited in the local lesions of the inoculated leaves but the viruses spread to the rest of the inoculated plant and caused systemic necrosis. Thus, the deposition of callose is not necessarily effective in restricting spread of viruses within plants.

Resistance to Virus Replication

Ross (1961a, 1961b) demonstrated that inoculation of half leaves of Samsun NN with TMV induced a high level of resistance to TMV in the opposite half leaves. Inoculation of lower leaves on a plant also induced resistance in upper leaves and vise versa. A virus-inhibitory substance, which was synthesized in the inoculated leaves and then moved to the rest of the plant, was proposed as the cause of resistance. Indications of a host-mediated process that suppressed virus replication in local lesion hosts were also obtained in studies using actinomycin D (Loebenstein, et al., 1969) and UV irradiation (Loebenstein, et al., 1970). Antiviral agents have been extracted from both infected and uninfected resistant tissues (Sela, et al., 1966; Mozes, et al., 1978). However, their role in virus localization or induced resistance is still an open question because these extracts were tested mainly as inhibitors of infection by mixing with the test virus before inoculation, or by application at short intervals (15 min to 2 hr) after inoculation (Antignus, et al., 1977). Loebenstein (1972) proposed that to be evaluated as inhibitors of replication, the test materials should be applied to the infected cells 5-12 hr (or more) after inoculation. Loebenstein and Gera (1981) found that a substance(s) inhibiting virus replication (IVR) is released into the medium

from TMV infected protoplasts of Samsum NN. IVR was not produced in protoplasts from susceptible plants nor from noninoculated protoplasts of the resistant cultivar. IVR inhibited virus replication in protoplasts from both susceptible and resistant varieties when applied up to 18 hr after inoculation. IVR was partially purified using zinc acetate precipitation and yielded two biologically active substances with molecular weights of about 26,000 and 57,000. Gera and Loebenstein (1983) in later studies reported that the IVR inhibited replication of TMV, CMV and potato virus X in leaf disks of different hosts, indicating that the inhibition of IVR is not specific to TMV . No IVR could be detected in the medium from protoplasts of Samsun NN inoculated with CMV, thus the induction of IVR is specific to TMV.

When cultivars of cowpea were evaluated for their resistance against the SB strain of cowpea mosaic virus (CPMV), three distinct types of responses, namely susceptible, resistant, and immune were detected (Beier, et al., 1977). The ability of protoplasts to support replication of CPMV was investigated by inoculation of mesophyll protoplasts with CPMV. Among 55 immune lines, protoplasts of 54 lines supported CPMV replication to levels similar to those of the susceptible varieties. Only one immune line, Arlington, supported significantly lower levels of virus

replication (1 percent of the level of susceptible line Blackeye 5).

Phenotypic expression of different genes controlling resistance to tobacco mosaic virus (TMV) in tomato was analysed using protoplasts of isogenic breeding lines (Motoyoshi and Oshima, 1977). Resistance genes Tm-2 and $Tm\text{-}2^2$ were not expressed and did not prevent infection and multiplication of TMV-L, a common tomato strain of TMV. By contrast, the homozygous resistance gene Tm-1 was able to express its effect in protoplasts as well as in leaf discs. Protoplasts and leaf discs homozygous for Tm-1, however, became infected with TMV-CH2, a tomato strain able to overcome the effects of Tm-1 in intact plants. Protoplasts of Lycopersicon peruvianum P.I. 128650, known to have a high level of resistance to TMV, were as readily infected with TMV-L, and synthesized progeny virus as rapidly as protoplasts of susceptible tomato.

TMV multiplies in isolated protoplasts of tobacco bearing the N gene to the same extent as in protoplasts obtained from cultivars in which TMV spreads systemically (Otsuki, et al., 1972). However, Loebenstein et al. (1979) reported that the multiplication of TMV in protoplasts of Samsun NN was markedly reduced if 2,4-dichlorophenoxyacetic acid was omitted from the protoplast medium. Thus the physiology of isolated protoplasts may be different

from cells of the intact plant. Judging from the above examples, the behavior of viruses in isolated protoplasts may not be the same as in cells of intact plants. Experiments using inoculation of isolated protoplasts must be interpreted carefully.

Mechanisms of MDMV Resistance

Tu and Ford (1970) reported that MDMV-A replicated to similar amounts in inoculated leaves of resistant (Illinois A) and susceptible (Seneca Chief) varieties but mosaic symptoms rarely developed in the resistant variety. Free amino acids increased in MDMV-inoculated leaves of both Illinois A and Seneca Chief, but the increase in amino acid concentration in newly emerged leaves was significant only in Seneca Chief, in which systemic virus movement occurred. They suggested that virus multiplication and metabolic alteration resulted in free amino acid accumulation. However the exact cause and consequences of such changes are not understood. Tu and Ford (1969) claimed that no inhibitor of virus replication was found in the resistant variety but their assay was ,in fact, detecting inhibition of infection because the inhibitor was added to the inoculum during inoculation. Jones and Tolin (1972) reported that the resistant corn hybrid T8 x 07B showed

only occasional longitudinal bands of chlorotic tissue in otherwise normal, dark green leaves in the summer. High concentrations of MDMV were extracted from the chlorotic bands but not from the dark green parts of the same leaves. They suggested that the mechanism of resistance in T8 x 07B is not against virus infection or multiplication, but is against movement of the virus in the host. The true mechanisms of resistance in maize against MDMV remain to be proven since other possibilities, such as synthesis of IVR were not investigated.

Spread of Virus in Tissues of Plants

Early work with viruses that can be mechanically inoculated to leaves showed that movement in systemically infected plants is of two kinds. First there is radial spread through the leaf away from the site of infection, and then, when a leaf vein is invaded, there is a directional spread through the vascular tissues to all parts of the plant (Samuel, 1931; 1934).

Many lines of evidence suggest that most viruses move through vascular tissue in the phloem rather than xylem. For example TMV does not pass from an infected part of a plant through sections of the stem killed by steam (Caldwell, 1931). Virus movement is usually closely corre-

lated with the direction and rate of movement of metabolites, and not with transpiration rate (Bennett, 1940; Cochran, 1946). By contrast southern bean mosaic virus seens to be naturally translocated in the xylem, infects the plant through the xylem, and moves through steamed sections of the stem (Schneider, 1965; Esau, 1967).

There is some evidence that the particle stage of the virus life cycle may be involved in spread within an intact plant. Siegel, et al., (1962) isolated a mutant of TMV that produces a defective coat protein which cannot assemble correctly with the genome RNA to form particles. The spread of this mutant within plants differs from that of normal strains of TMV in that it seems to be unable to enter and spread through the vascular system. It spreads slowly through a plant, usually infects only the leaves above the inoculated leaf on the same side of the plant one at a time and it never spreads directly to the tip leaves. Thus it seems that, for TMV at least, the uncoated genome RNA can spread from cell to cell through plasmodesmata, whereas only intact particles can spread through the phloem.

Shalla, et al., (1982) showed that a temperature-sensitive mutant of TMV (LS1) replicated and moved from cell to cell in intact tobacco leaves at 22 C but was restricted in movement when leaves were maintained at 32 C.

Electron microscopy of thin sections revealed a significantly lower number of plasmodesmata between LS1-infected palisade and/or mesophyll cells from leaves held at 32 C than those held at 22 C. The dynamic changes in number of plasmodesmata provide another possible mechanism of resistance in plants against viruses. Leonard and Zaitlin (1982) found that in vitro translation products of LS1 RNA included a protein which differs slightly from the 30,000 molecular weight protein translated from RNA of the L strain. This suggests that the 30,000 molecular weight protein may have a role in cell-to-cell movement of the L strain and when altered becomes non-functional.

Maize Dwarf Mosaic Virus

MDM was first observed in southern Ohio in the early sixties (Williams and Alexander, 1963). The symptoms appeared as leaf mosaic patterns, dwarfing, shortened internodes, ears with kernels missing, small ears, or complete lack of ear formation. The disease subsequently spread throughout most of the United States and southern Canada. MDM causes severe losses in dent corn and even more severe in sweet corn (Gordon, et al., 1981). MDMV exists as many strains but strains A and B are the most widespread. Strain A infects Johnsongrass (Sorghum halepense) while

strain B cannot (Mackenzie, et al., 1966). Louie and Knoke (1975) reported that differences among johnsongrass-infecting isolates from southern Ohio were sufficient and stable enough to warrant description of four new strains designated MDMV-C, D, E, and F. The johnsongrass-infecting strains (A, C, D, E, F) were differentiated by symptomatology, by the differential responses of selective corn inbreds to infection, and by differences in transmission efficiencies by several aphid species.

The particle of MDMV is filamentous and measures about 700-755 nm in length and 12-16 nm in diameter (Pirone, 1972; Thompson and Thornberry, 1968). Ultrastructural features of MDMV-infected cells include pinwheel inclusions composed of curved laminar arms that radiate from a central axis (Gardner, 1969; Kuhn and Kozelnicky, 1968; Langenberg and Schroeder, 1973). These characteristics classify MDMV in the potyvirus group of plant viruses (Edwardson, 1974; Pirone, 1972). The host range of MDMV includes 82 genera of Gramineae, predominating among the nonfestucoid grasses (Rosenkranz, 1978, 1980; Watson and Gibbs, 1974). The most important hosts are corn, sorghum, and johnsongrass. Corn and sorghum are the economic hosts most severely damaged by the virus (Toler and Bockholt, 1968), and johnsongrass is an important overwintering host for all strains except MDMV-B (Louie and Knoke, 1975;

Mackenzie, 1967; Mackenzie et al. 1966).

Genetics of MDM Resistance

A thorough review of the genetics of MDM resistance has been made by Gene, et al. (1981). They point out that some earlier studies which used symptomatology and depended on natural infection, may have been actually measuring maize chlorotic dwarf instead of MDM. Therefore, only studies which were conducted under situations free from doubt of interference will be described.

Scott and Rosenkranz (1981) evaluated the diallel crosses among three resistant and three susceptible corn inbred lines. The mean percentage of diseased plants for the resistant (R) x susceptible (S) crosses were nearer the mean for the S x S crosses, suggesting a degree of dominance for susceptibility. Utilizing chromosomal translocation, Scott and Nelson (1971) found that inbred GA209 had a gene for resistance to MDM on both arms of chromosome 6. Later, Scott and Rosenkranz (1973) reported that inbreds Ark H-24, Ark H-77, Mp 339, Mp 412, Mol 18W, and probably Tx 601 also had a gene for resistance on both arms of chromosome 6.

Scheifele and Wernham (1968) and Wernham and Scheifele (1969) suggested that in at least one inbred, resistance to

MDMV-A was conditioned by a single dominant gene and not by the same gene that conditioned resistance to MDMV-B. Scott and Rosenkranz (1977; 1981) evaluated a diallel cross by manually inoculating with MDMV in the field and found that the R x S crosses were intermediate between the R x R and S x S crosses. This suggests an additive type of gene action for resistance. Findley, et al. (1977) reported that resistance to MDMV-B in inbred Pa405 appeared to be controlled by two dominant genes when F2 seedlings from the cross Pa405 x Oh28 were mechanically inoculated and a single dominant gene when plants were exposed to inoculation by viruliferous aphids. Also, resistance seems to be due to a single dominant gene for mechanically inoculated F2 plants of M14 x Pa405.

Scott and Rosenkranz (1982) proposed a new method for genetic studies of MDM resistance. Under the assumptions that genes are independent, absence of dominance, and that each allele has an equal effect, frequency of diseased plants in a certain segregating generation can be predicted for a given number of alleles for resistance. They found that inbreds Ga209, Mp339, Mp412, T240, and Va35 had 2, 2, 2, 3, and 1 genes for resistance,respectively, to MDMV-A. Ga209 probably has one gene for resistance in common with Mp339. The gene for resistance in Va35 is different from both genes in Mp339.

Mikel, *et al*. (1984) showed that disease incidence in F2 segregates of Su x Pa405 fit a 45R = 19S ratio of a three gene model where one gene must be present with either of the other two. The testcross segregates fit a 3R =5S ratio for the same three gene model. F2 segregates of Su x B68 fit a 27R = 37S ratio of a three gene model where all three genes must be present for MDMV resistance. Testcross segregates in the same year fit a 1R = 7S ratio of the same three gene model.

Johnson (1971) used diallel crosses to analyze the genetic basis of MDM resistance. General combining ability variance accounted for most of the variation among crosses; resistance appeared to be usually inherited with nearly complete dominance, and midparent heterosis for resistance was observed. He suggested that variation from minor gene effects was usually additive and was responsible for a large propotion of the variation among crosses.

Monitoring Viral Antigen with Immunofluorescense

The fluorecent antibody technique, originated by Coons, *et al*. (1942), has been used extensively in various aspects of virus research. The technique is readily applicable in most animal systems. However, due to the difficulty with which antibodies penetrate plant tissues and the

interference by autofluorescing plant materials, the technique must be modified for application in plant tissues.

Plant materials have been prepared for fluorescent antibody staining by thin sectioning of tissues. Frozen sections have been used with success (Schramm and Rottger, 1959; Margaretha, et al.,1964). Worley and Schneider (1963), however, found that the configuration of frozen sections of bean leaves can best be preserved when the sections are lyophilized and fixed with chloroform. Nagaraj (1962) developed a method in which tobacco leaf cells were dissociated by digestion with pectinase and TMV antigen could then be stained with fluorescent antibody in the unfixed, dissociated cells. Separation of single cells eliminated the interference of the autofluorescence and facilitated the penetration of antibody. Nagaraj (1965) further compared different ways of preparing speciments for immunofluorescent staining. He found that, when the pectinase digestion method is used, only infected cells from older leaves can be stained while infected cells from younger leaves can not be stained unless the cells are fixed. He concluded that the pectinase digestion method was very useful in detecting small quantities of antigens while paraffin sectioning gave less consistent results because only small areas may be sectioned and observed.

However, the resolution of the intracellular antigen in the paraffin sections was better than that in the dissociated cells. Paraffin sections also enable the detection of viral antigen in various types of plant tissues while the pectinase digestion method can only be used on mesophyll cells. Otsuki and Takebe (1969) developed a fluorescent antibody staining method for detection of TMV antigen in tobacco mesophyll protoplasts. The protoplast method enables fine resolution of the intracellular antigen.

Hosokawa and Mori (1974) found that, if epidermal cells of cucumber leaves were first treated with cellulolytic enzymes, the intracellular viral antigen of the epidermal cells can be stained with fluorescent antibody. Nishiguchi, et al. (1980) adopted a similar method to analyze the profile of TMV spread in epidermis of tomato.

C H A P T E R III

MATERIALS AND METHODS

Virus and Plant Materials

The B strain of MDMV (MDMV-B), kindly supplied by Dr. R. E. Ford, of the University of Illinois, was used throughout these experiments. Two MDM-resistant inbred varieties, PA405, a field corn, and BSQ, a sweet corn, and a MDM-susceptible sweet corn inbred ,MA5125, were used in all experiments unless otherwise stated. BSQ is the product of a cross between B68 and Silver Queen. In some experiments two F1 hybrids, PA405 x MA5125 and MA5125 x BSQ were also used. The initial seeds of all corn lines and hybrids were kindly provided by Dr. David MacKenzie formerly of the University of Massachusetts, now with Harris Seed Co. Rochester, NY. Plants were grown in the greenhouse in 6-inch pots in a 1:1:1 sand, peat, and soil mixture amended with lime, at temperature maintained generally near 25 C, but occasionally reaching 30 C during day and 20 C during night. All plants were kept in the greenhouse until use. MDMV-B was maintained in young Golden Cross Bantam plants. Young leaves with mosaic symptoms were used as inoculum after grinding in inoculation buffer (0.05 M Na-phosphate,

10 mM NaDIECA, pH 7.4) at a ratio of 1:10 (W/V). Carborundum was added to virus sap which was then rubbed onto plants with cotton swabs. The first fully opened leaves of plants at the 4-leaf stage were inoculated throughout the experiment unless otherwise stated. The plants were ramdomized during inoculation.

Serological Test

Preparation of Antibody

MDMV-B was purified by the procedure of von Baumgarten and Ford (1981). At weekly intervals, rabbits were given 3 subcutaneous and then 1 intravenous injections of 0.5 mg purified MDMV-B. The rabbits were bled one week after the last injection. The serum had a titer of 256 as determined by microprecipitin test. To avoid non-specific staining of healthy tissue, the antiserum was routinely cross-adsorbed with healthy tissue before use. In order to remove the non-specific antibodies, each ml of antiserum was mixed with 0.1 g of healthy corn leaf powder that had been extracted and decolorized with acetone. The mixture was incubated at 37 C for 1 hr and centrifuged. The supernatant was used as the antiserum in ELISA tests. Two more adsorptions were carried out if the antiserum was to be used for immunofluoresent staining. The antibody was then precipi-

tated with 50 % ammonium sulfate dissolved in 1 ml of phosphate-buffered saline (PBS) pH 7.4 containing 0.2 % sodium azide, and dialyzed against 3 changes of 500 ml PBS. The partially purified antibody was used in immunofluorescent staining.

Enzyme-Linked Immunosorbent Assay

The double sandwitch method of enzyme-linked immunosorbent assay (ELISA) was performed by the method of Clark and Adams (1977) with minor modifications. Antibody was purified from the adsorbed antiserum by precipitation with 50 % ammoniun sulfate and then passing through DEAE celullose column. The concentration of MDMV-B antibody used for coating of wells of specially coated polystyrene microtiter plates was adjusted to 1 ug/ml. The alkaline phosphatase conjugated antibody was diluted 1 : 1,000. Leaf samples were ground in virus buffer (PBS with 0.05 % Tween 20, 2 % polyvinylpyrolidone and 0.2 % ovalbumin). The above conditions were used routinely and will be referred to as the standard ELISA test.

Immunofluorescent Staining

The lower epidermis of a 4 cm section of the virus-inoculated leaf was brushed with an artist's water-color brush dipped in a slurry of carborundum in distilled water.

The leaf sections were brushed 100-200 times in each direction. Older leaves required more brushing to permit efficient penetration and digestion of the tissue by the enzymes. The brushed leaves were rinsed with distilled water to remove the carborundum. Three leaf pieces were incubated with the brushed side down in 5 ml of enzyme solution (2% Cellulycin, 1% Driselase, 0.5 M mannitol, 10 mM CaCl ,pH 5.5) in a Petri dish. This and all subsequent incubations were carried out on a rotary shaker (20 rpm) at room temperature. A uniform water-soaked appearance over the entire leaf section signaled completion of digestion. Digestion was usually completed in about 40-60 min. The enzyme solution was removed and the leaves were washed with 2 changes of PBS at 5 min interval. The PBS was drained and acetone was added to the dish for 40-60 min to fix and decolorize the tissue. The fixed leaves were washed with 3 changes of PBS at 30 min intervals to remove the acetone. The fixed leaves were stored in PBS at 4 C until further processing. Two ml of MDMV-B antibody of O.D.280 = 0.01 were added per dish of leaf sections. After 12-24 hr of incubation the leaves were washed with 3 changes of PBS over a period of 24 hr. Three ml of appropriately diluted fluorescein isothiocyanate-conjugated anti-rabbit antibody developed in goat (Sigma Chemical Co. St. Louis, MO) was added to the leaves which ,after 12-24 hr of incubation at

room temperature, were washed overnight with three changes of PBS. After the last wash the leaves were floated in PBS and were lifted out flat on microscope slides. Excess PBS was allowed to run off and the leaves were mounted in 10 % glycerin in PBS. The stained leaves were observed with a Leitz Dialux 20 epifluorescent microscope with the Leitz K2 and L2 filter systems which have an extremely narrow band BLUE at 495 nm and a selective barrier at 525 nm, specific for fluorescein isothiocyanate (FITC) and a few other stains. Photographs of fluorescent sections were taken with Kodak Ectachrome, ASA-400 film with exposure times of 30-60 sec.

Determination of Resistance of Sweet Corn Varieties

Fifty two inbred varieties of sweet corn (names listed in results), kindly supplied by Dr. G. Hochmuth of the University of Massachusetts, were used in these experiments. Four-cm sections of fully expanded leaves were inoculated. The inoculated plants were examined daily for symptom expression up to 30 days. Plants which remained symptomless 30 days after inoculation were considered resistant. The inoculated areas of the symptomless plants were tested with the standard ELISA procedure for presence of MDMV-B.

Field Evaluation of MDM Resistance

Plants of the three inbreds and two F1 hybrids were inoculated at the 5-leaf stage. The first experiment was carried out during June-September, 1982. A completely randomized plot design was adopted. Each plot consisted of 10 plants of one variety planted in a row. The treatments were one inoculation of MDMV-B at the 5-leaf stage, 4 inoculations of MDMV-B at weekly intervals, and uninoculated control. The inoculation was carried out by rubbing the first three young leaves with virus sap. There were two plots for each treatment and the total of 30 plots were completely ramdomized.

The second experiment was carried out during June-September, 1983. A ramdomized block design was adopted. The treatments were two inoculations at 7-day interval starting at the 5-leaf stage, and uninoculated control. A total of 10 plots were completely randomized in a block. Five blocks were arranged parrallel to each other. Plants were evaluated weekly for disease incidence and severity.

Infectivity of MDMV-B from Resistant Leaves

The inoculated leaves were concurrently tested for virus concentration with ELISA and for virus infectivity by

bioassay. The inoculated leaves were collected 7 days after inoculation and ground in 0.05 M phosphate buffer pH 7.4 at a ratio of 1:5 (W/V). In tests of virus concentration the saps were diluted 10-fold in virus buffer and assayed with the standard ELISA procedure. In infectivity tests, saps were diluted 10-fold in inoculation buffer and were inoculated onto 25 seedlings of Gold Cup. Percentages of diseased plants were recorded 30 days after inoculation. ELISA values were then compared with percentages of diseased plants to determine whether the virus in the resistant leaves was infectious or inactive.

Test for Aquired Resistance

In the first experiment, 4-cm leaf sections were inoculated with MDMV-B to elicit the production of a potential antiviral substance. The emerging new leaves were challenge-inoculated 7 days after the first inoculation. In the 2nd and 3rd experiments, challenge inoculations were made on new leaves plus leaf sections adjacent to the area of inoculation. One set of MDMV-B inoculated plants was left unchallenged to determine the extent of virus spread. In the 4th experiment, plants inoculated with healthy sap were challenged as additional control. In all experiments, 4-cm leaf sections were ground in one ml of virus buffer and the

concentration of viral antigen was measured with the standard ELISA procedure.

Plants were also treated with actinomycin D to determine whether the resistance mechanism(s) involved active host response after infection. Seeds were planted in a mixture of vermiculite and peat (1:1) and were fertilized by saturating the mixture with the Ruakura solution of Smith, et al. (1983). Plants were watered daily with distilled water. Two-cm sections of a fully opened leaf of a plant were inoculated. Twenty four hours after inoculation the inoculated plants were carefully removed from the planting mixture and the roots were washed in distilled water to remove peat and vermiculite. Five plants in a group were allowed to absorb 10 ml of an actinomycin D solution (50 ug/ml in distilled water) in a dark growth chamber. It took about 24 hrs for complete uptake of actinomycin D. The treated plants were then replanted in a fresh peat-vermiculite mixture and were incubated for 6 days in a growth chamber with 16 hr of day period at 27 C. In the first two experiments, two-cm leaf sections were collected from the area of inoculation, the adjacent sections, and the new leaves. Each leaf section was ground in one ml of virus buffer for the ELISA test. In the third experiment, MDMV-B was inoculated onto a 2-mm strip across a leaf. Six-cm long leaf sections, 4-cm from the inocula-

tion strip towards the proximal end and 2-cm from the inoculation strip toward the distal end, were collected 6 days after inoculation and examined with the immunofluorescent procedure. The youngest new leaves were also tested with ELISA.

Determination of Number of Infection Loci

Four-cm leaf sections were inoculated on the upper epidermis as before. The inoculated sections were collected at approximately 12 hr intervals up to 68 hr after inoculation. Leaf samples were stained with fluorescent antibody and observed as described. The entire surface of a leaf section was scanned with the fluorescent microscope and single fluorescent cells or groups of fluorescent cells representing different infection loci were counted. The numbers of infection loci were adjusted for the width of leaves to give the number of loci per square cm. The first two experiments were carried out during January of 1984 and after inoculation the plants were incubated in a growth chamber at 27 C with 16 hr day period. The third experiment was carried out during April of 1984 and the plants were kept in the greenhouse throughout the experiment.

Determination of Rate of Cell-to-Cell Spread of MDMV-B

The same samples used for determination of the number of infection loci were used for determination of the rate of cell-to-cell spread of the virus. The length of each infection locus, i.e. it's dimension parallel to the veins of the leaf, was measured with an occular micrometer. All ,or up to 10 random loci were measured. The average length of the infection loci on one leaf was taken as a unit in the statistical analysis.

Determination of the Rate of Long-Term Spread

Two different assays were used to determine the rate of long-term spread of MDMV-B in corn. In experiments using ELISA, 4-cm leaf sections were inoculated. The three inbreds and the two F1 hybrids were used in these experiments. Four-cm leaf sections were collected at 3-day intervals from the areas of inoculation, from the adjacent sections, and from the newest leaf of the plants. Each leaf section was ground in one ml of virus buffer and assayed with the standard ELISA procedure. In experiments using the immunofluorescent staining technique, MDMV-B was inoculated in 2-mm strips perpendicular to the leaf axis of the upper epidermis of the leaf. Only the inbreds were

used in these experiments. Leaf sections 4-cm long, containing the inoculated strip in the middle of the section, were collected at 2- or 3-day intervals. After immunofluorescent staining of the leaf sections, the inoculated strips were first examined to locate the primary infection loci. When a primary infection locus was found, its expansion was traced along the veins on either side of the locus. The distance from the farthest point of distal (leaf tip) spread to the farthest point of proximal (leaf base) spread was measured with an ocular micrometer or the stage meter of the microscope whichever was more appropriate. If more than one primary infection loci were present, the average distance of spread was taken as one observation. The uninoculated areas of the leaf sections were then scanned with the fluorescent microscope for any secondary infection loci which could not be traced back to a specific primary infection locus along one of the veins. The experiment was repeated three times.

Comparing Distal Versus Proximal Spread

Samples from two of the experiments in which the rate of long-distance spread was determined by immunofluorescent staining were used in this comparison. The average distances from the primary infection loci to the farthest distal

and farthest proximal point where virus could be detected were compared.

Statistical Analysis

Statistical analysis of the data was carried out by using the general linear model procedure (PROC GLM) computer program of the Statistical Analysis System (SAS Institute Inc., SAS circle, P.O. Box 8000, Cary, NC 27511-8000). In all cases a fully interactive model was chosen. In such a model, the dependent variable Y is given by the mathematical formula Y=aV1+ bV2 + cV1 V2, in which, V1 and V2 are independent variables, V1 V2 is their interaction and a, b, and c are constants. If the variance component due to a specific variable (e.g. variety, time) was significant, its levels were further compared by Duncan's multiple range test with significance level p = 0.05. If the interaction between variables was significant, it was further analysed by the procedure of least square means. Only the comparisons of interest with total p=0.05 were examined (if 10 comparisons were made,the significance level would be 0.005).

C H A P T E R IV

RESULTS

Immunofluorescent Staining Technique

Under ultra-violet light, the acetone-fixed, decolorized and antiserum-treated healthy leaves produced a red fluorescence of low intensity, while similarly treated virus infected cells produced a bright apple-green fluorescence. All treated mesophyll cells were in a state of plasmolysis and thus appeared to be round. Some epidermal cells in the area of inoculation, which were injured during mechanical inoculation with either healthy or diseased sap, adsorbed fluorescent dye and their entire cytoplasm became uniformly and brightly fluorescent (Fig. 1). The non-specifically stained cells, however, could be easily differentiated from virus infected cells because the fluorescence of the latter appeared to be characteristically more concentrated in some area of the cytoplasm than in others (Fig. 2). Non-specific staining increased when leaves were severely injured during inoculation.

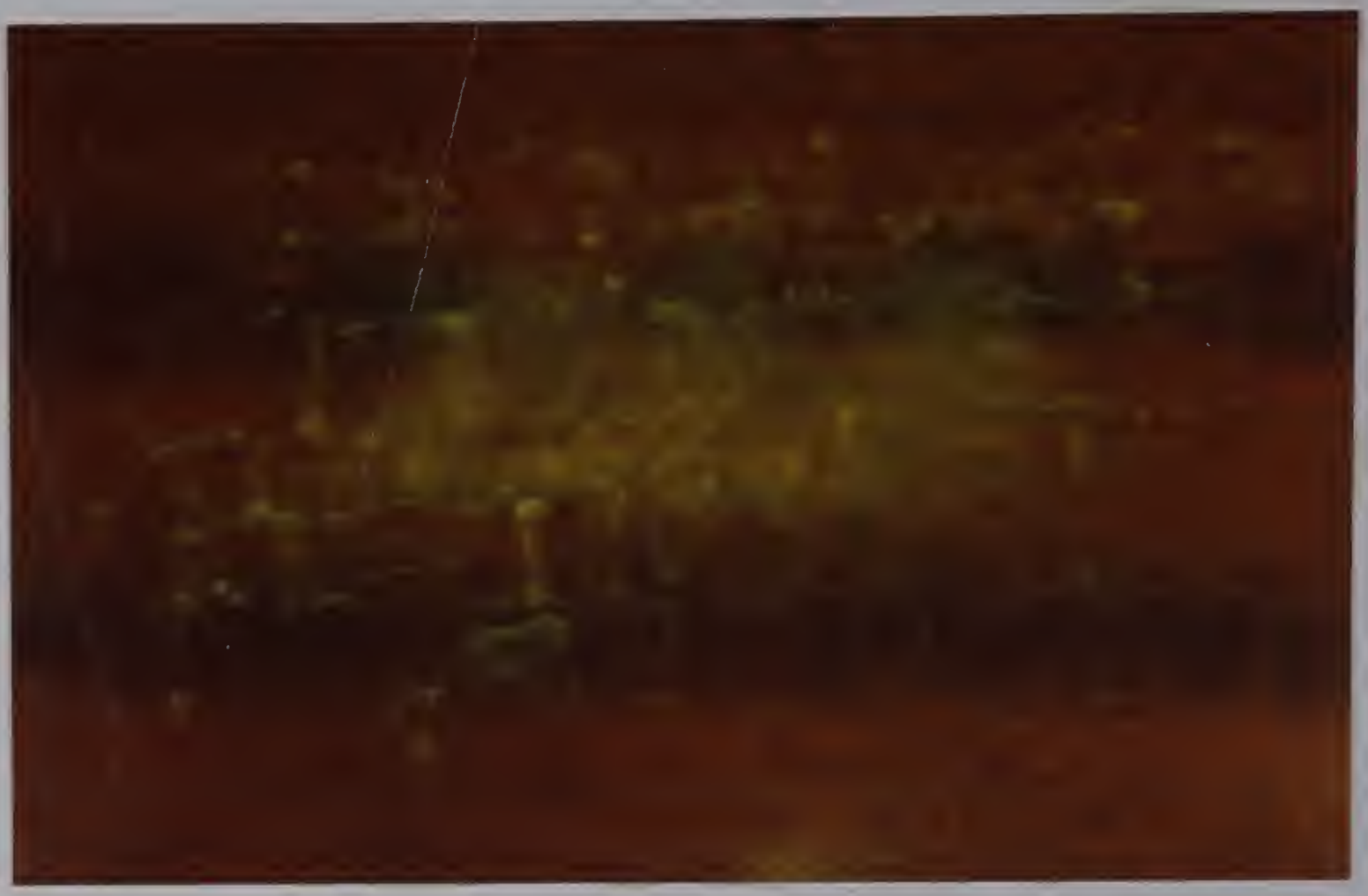
The epidermal cells and 1-2 layers of mesophyll cells could be clearly observed with epifluorescent microscopy (Fig. 3). At later stages of infection, when the number of

Fig. 1. Uninfected cells fluorescing brightly over their entire surface as a result of mechanical injury during inoculation. Such injured cells non-specifically adsorbed fluorescent dye during preparation of fluorescent staining.

Fig. 2. Viral antigen, distributed irregularly in the cytoplasm of Ma5125 cells 48 hrs after inoculation, appears as specks of bright green fluorescence after immunofluorescent staining.

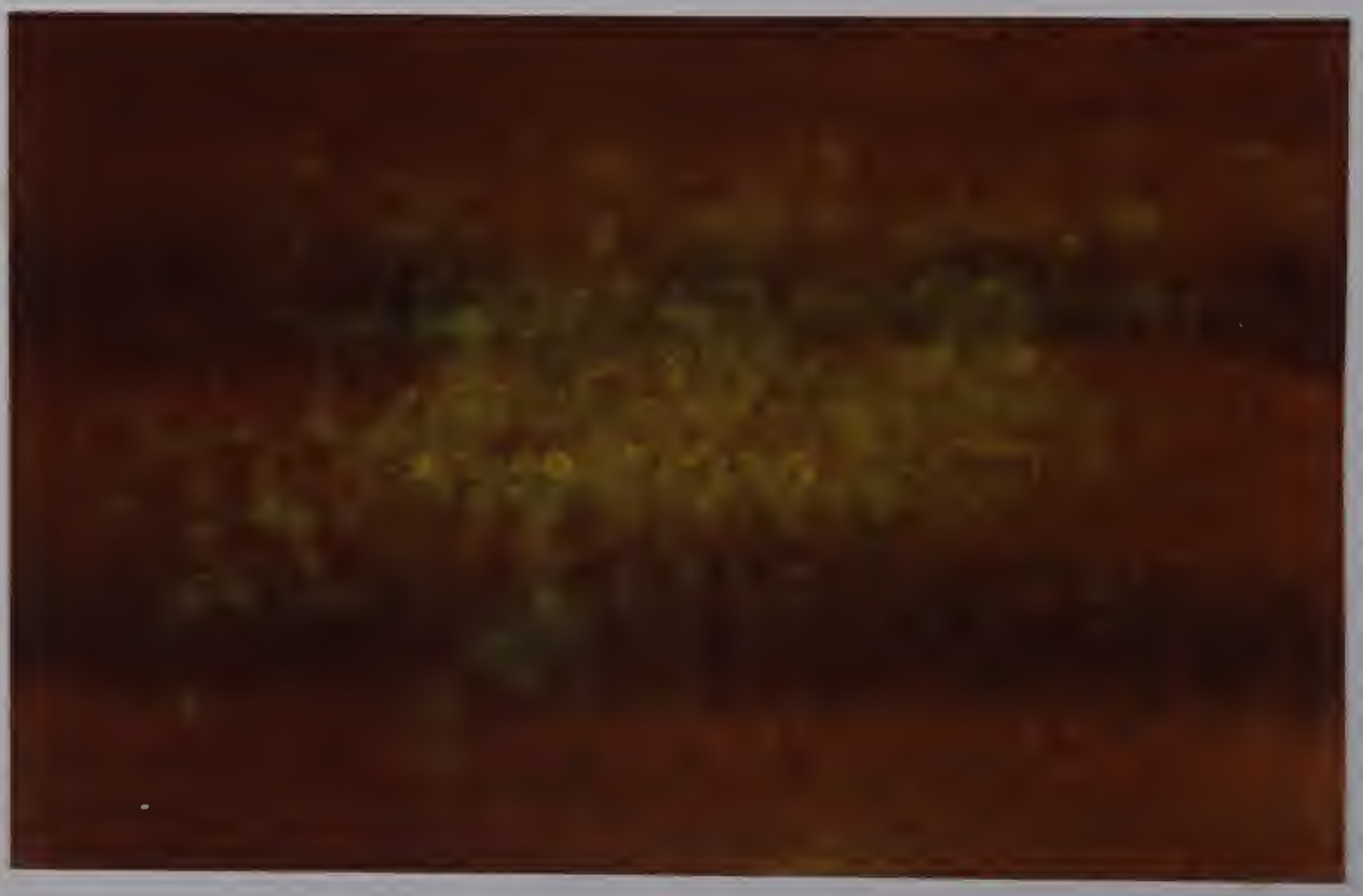
Fig. 3. Groups of virus-infected epidermal (a) and mesophyll (b) cells in inoculated Pa405 leaves 44 hrs after inoculation. Viral antigen accumulated mostly at the periphery of infected epidermal cells (a).

a



0.1 mm

b



infected cells increased, the fluorescence of infected cells in the upper layer interferred with the fluorescence of infected cells in deeper layers, thus the infected cells in the lower layer became difficult to observe. Cells of the bundle sheath and of the vascular system were beyond the resolution of this method. Fluorescence in infected cells was first observed on the periphery of the rounded protoplasts 24 hr after inoculation (Fig. 4). Specks of bright green fluorescence developed irregularly in the cytoplasm 48 hr after inoculation (Fig. 2).

Screening of Sweet Corn Varieties for Resistance

Thirteen of 52 sweet corn inbreds tested were resistant to MDMV-B (Table 1,2). All 13 resistant sweet corn varieties were derivatives of B68, a resistant field corn variety. The susceptible varieties showed mosaic symptoms 5-14 days after inoculation. Plants of the resistant varieties remained symptomless throughout the experiment. However, when the inoculated leaves were assayed with ELISA, high titers of viral antigen were found (Table 2).

Field Evaluation of MDMV-B Resistance

Pa405, Bsq, and Pa405 x Ma5125 were highly resistant

Fig. 4. Virus infected cells (3 in a, 12 in b) in inoculated leaves of Pa405 (resistant variety), observed 24 hrs after inoculation. After immunofluorescent staining, viral antigen appeared first as green fluorescence at the periphery of the protoplasts.

a

10 u

b

Table 1. List of Sweet Corn Varieties Inoculated with MDMV-B and Their Reaction to the Virus.

VARIETY	D/T[a]		VARIETY	D/T	
21547-1-PA	9/9	S[b]	2412-1	8/8	S
2256 B	4/4	S	51.346	6/6	S
51408 x A	9/9	S	51408 x B	8/8	S
51408 x C	3/3	S	51408 x D	10/10	S
63.283-76	8/8	S	69.36	10/10	S
70-152	10/10	S	76-32	8/8	S
76-34	11/11	S	76-35	6/6	S
9E-79-15	10/10	S	B1002-73	10/10	S
C7	8/8	S	C5NT	23/30	S
C27	8/8	S	Cr13	10/10	S
Cx13	8/8	S	IL671a	10/10	S
IL677a	10/10	S	J842	9/9	S
M6465-132	10/10	S	M6466-133	10/10	S
MA5125	10/10	S	Me200	10/10	S
Me2rt	10/10	S	NJ501	10/10	S
P39LE	11/11	S	V611	26/26	S
V631	24/24	S	V641	10/10	S
V642	8/8	S	V726	5/5	S
W6053	10/10	S	W6765st	22/22	S
Bsq	0/21	R	Bst-1	0/17	R
Bsq-1	0/18	R	Bst-2	0/15	R
Bsq-2	0/19	R	Bst-3	0/26	R
B68SCC-1	0/26	R	Bst-4	0/20	R
B68SCC-2	0/25	R	B68SCC-5	0/21	R
B68SCC-3	0/15	R	B68SCC-6	0/24	R
B68SCC-4	0/17	R			

[a] D/T = diseased plants/total plants inoculated.

[b] S = susceptible, R = resistant.

Table 2. Titer of MDMV-B in Inoculated Leaves of Resistant Sweet Corn Varieties.

VARIETY	EXPERIMENT 1ST	2ND
BSQ	1.163[a]	1.009
BSQ-1	1.079	.924
BSQ-2	1.151	.942
BST-1	.572	.679
BST-2	.506	.861
BST-3	.824	.743
BST-4	.843	.628
B68SCC-1	.985	.576
B68SCC-2	.786	.900
B68SCC-3	.307	.606
B68SCC-4	.925	.476
B68SCC-5	.679	.826
B68SCC-6	.732	.765

[a]average ELISA values at O.D.405 for 5-10 plants.

to MDMV-B (Table 3,4,5,6). No plants of these three varieties ever showed any symptom of MDMV infection. However leaf discs collected from inoculated areas of the leaf produced high concentration of viral antigen as measured by ELISA. The hybrid Ma5125 x Bsq appeared to be intermediatly resistant in the 1982 experiment (Table 3,4) when the summer was unusually cool. The same hybride, however, appeared highly resistant in the 1983 experiment (Table 5,6) when the summer was hot. Symptoms on Ma5125 x Bsq plants consisted of narrow bands of chlorotic tissue in otherwise dark green leaves. Leaf discs collected from chlorotic tissue produced high titers of virus when tested with ELISA. Leaf discs collected from dark green areas appeared to be virus free. In both experiments, all plants of the susceptible variety Ma5125 developed symptoms (Table 3,5) and their disease severity rating was the highest possible (Table 4,6). The uninoculated control plants never developed symptoms. When young leaves of inoculated plants were tested with ELISA at tasselling stage, MDMV-B was detected in new leaves of Ma5125 and Ma5125 x Bsq but never of Pa405, Bsq, and Pa405 x Ma5125.

Infectivity of MDMV-B from Resistant Leaves

In the first experiment, in which leaf samples were

Table 3. Incidence of Maize Dwarf Mosaic Symptoms in Inoculated Plants of Corn Varieties of Differential Resistance in a 1982 Field Experiment.

VARIETY	NUMBER OF INOCULATIONS	WEEK AFTER INOCULATION 1	2	3	4
Pa405(Pa)	1	0[a]	0	0	0
	4	0	0	0	0
Bsq	1	0	0	0	0
	4	0	0	0	0
Pa x Ma	1	0	0	0	0
	4	0	0	0	0
Ma x Bsq	1	0	40	54	72
	4	0	35	60	80
Ma5125(Ma)	1	100	100	100	100
	4	100	100	100	100

[a]percent symptomatic plants.

Table 4. Disease Severity in MDMV-B Inoculated Plants of Corn Varieties of Differential Resistance in a 1982 Field Experiment.

VARIETY	NUMBER OF INOCULATIONS	WEEK AFTER INOCULATION 1	2	3	4
Pa405(Pa)	1	0[a]	0	0	0
	4	0	0	0	0
Bsq	1	0	0	0	0
	4	0	0	0	0
Pa x Ma	1	0	0	0	0
	4	0	0	0	0
Ma x Bsq	1	0	0.68	1.13	1.31
	4	0	0.35	1.15	1.35
Ma5125(Ma)	1	2	3.63	4.0	4.0
	4	2	3.42	4.0	4.0

[a] disease severity index scale from 0-4, 0=no symptoms, 1=mosaic confined to narrow bands on the upper one or two leaves, 2=mosaic covering entire surface of one or two upper leaves, 3=mosaic covering the entire surface of three to six of the upper leaves, 4=mosaic covering entire surface of most of the leaves of the plants.

Table 5. Incidence of Symptoms in MDMV-B Inoculated Plants of Corn Variety of Differential Resistance in a 1983 Field Experiment.

	WEEK AFTER INOCULATION			
VARIETY	1	2	3	4
Pa405(Pa)	0[a]	0	0	0
Bsq	0	0	0	0
Pa x Ma	0	0	0	0
Ma x Bsq	0	6	6	6
Ma5125(Ma)	100	100	100	100

[a] percent of symptomatic plants.

Table 6. Disease Severity in MDMV-B Inoculated Plants of Corn Varieties of Differential Resistance in a 1983 Field Experiment.

VARIETY	WEEK AFTER INOCULATION 1	2	3	4
Pa405(Pa)	0[a]	0	0	0
Bsq	0	0	0	0
Pa x Ma	0	0	0	0
Ma x Bsq	0	0.006	0.012	0.012
Ma5125(Ma)	2	4	4	4

[a] Disease severity index scale from 0-4, 0=no symptom, 1=mosaic confined to narrow bands on the upper one or two leaves, 2= mosaic covering entire surface of one or two upper leaves, 3=mosaic covering the entire surface of three to six of the upper leaves, 4= mosaic covering entire surface of most of the leaves of the plants.

collected 7 days after inoculation, the highest dilution that yielded positive readings in ELISA and the dilution-end-point of virus infectivity were both 1:1,000 in all varieties (Table 7). In the second experiment, leaf samples were collected 12 days after inoculation and, in all varieties, the highest dilution that gave positive readings in ELISA was 1:10,000, while, the dilution-end-point of virus infectivity was 1:100. Therefore, the viral antigen in the inoculated leaves of all varieties increased while the infectivity of the virus in all varieties decreased.

Tests to Determine Aquired Resistance

To test for potential mechanism(s) of aquired resistance, resistant plants were first elicited by inoculation with either virus sap or healthy sap and 7 days latter the elicited plants were challenge-inoculated with the virus. Seven days after challenge inoculation, plants elicited with sap from virus infected plants produced virus titer higher than or similar to that of plants elicited with healthy sap (Table 8). In an experiment in which unelicited plants were used as control, upon challenge inoculation the ellicited plants also produced virus titer that was higher than or similar to that of the unelicited plants (Table 9). In two experiments some plants inoculated with MDMV-B were

Table 7. Ralative Titer of Viral Antigen and Virus Infectivity in Corn Varieties of Differential Resistance.

VARIETY	DAY AFTER INOCULATION	ELISA	BIOASSAY
Pa405	7	1,000[a]	1,000
Bsq	7	1,000	1,000
Ma5125	7	1,000	1,000
Pa405	12	10,000	100
Bsq	12	10,000	100
Ma5125	12	10,000	100

[a]dilution end point.

Sample was pooled from 10 leaf sections of 5 plants collected 7 or 12 days after inoculation in two separate experiments.

Table 8. ELISA Values of MDMV-B Titer in Leaves of the Resistant Variety Pa405 after Challenge Inoculation of Previously Inoculated Plants.

INOCULATION		LEAF SECTION				
1ST	2ND	INOC[a]	PROX	DIST	NL1	NL2
VIRUS	VIRUS	1.31[b]A[c]	1.19 A	1.21 A	.70 A	.79 A
VIRUS	NONE	1.28 A	.75 B	.65 B	NT[d]	NT
HS[e]	VIRUS	NT	.82 B	.82 B	.22 B	.26 B

[a] INOC, leaf sections of inoculation, PROX, adjacent proximal section of the inoculated leaf section, DIST, adjacent distal section of the inoculated leaf section, NL1, 1st new leaf, NL2, 2nd new leaf.

[b] ELISA values at O.D.405.

[c] numbers followed by the same letter are not significantly different.

[d] NT, not tested.

[e] HS, healthy sap.

Experiment performed once with 8 plants per treatment.

Table 9. ELISA Values of MDMV-B Titer in Leaves of Two Resistant Varieties after Challenge Inoculation with MDMV-B of the New Leaves of Previously Inoculated Plants.

PREVIOUS INOCULATION	VARIETY PA405	VARIETY BSO
VIRUS	.232[a] A[b]	.601 A
NONE	.245 A	.331 B

[a] ELISA values at O.D.405.

[b] numbers followed by the same letter are not significantly different.

Experiment performed once with 10 plants per treatment.

not challenge-inoculated in order to determine the extent of spread of the virus from the first inoculation in relation to virus spread following challenge inoculation. Leaf sections immediatly outside the presumed front of virus spread (as indicated by the virus spread in unchallenged leaves) also produced high virus titer after challenge inoculation (Table 10).

In some experiments, plants were treated with either actinomycin D (treated plants) or water (control plants) to determine the effect of actinomycin D on spread of virus in plants. In the resistant varieties, spread of the virus was identical in actinomycin D treated plants and control plants (Table 11). MDMV-B did not move to the new leaves of the resistant plants in both treatments. The virus did not move into the leaf sections adjacent to the inoculated sections in Pa405 while it did in Bsq. In the susceptible variety, MDMV-B spread throughout the inoculated plants in both actinomycin D treated plants and control plants.

In one experiment, virus spread was monitored by the immunofluorescent technique. The distance of virus spread in treated plants was not significantly different from that in control plants (Table 12). MDMV-B was also not found in the new leaves of the resistant varieties in both treatments. In the susceptible variety, MDMV-B spread to the edges of the leaf sections (3 cm) in all samples.

Table 10. ELISA Values of MDMV-B Titer in Leaves of Two Resistant Varieties after Challenge Inoculation of Previously Inoculated Plants.

	INOCULATION		LEAF SECTION					
VARIETY	1ST	2ND	INOC[a]	0-4CM[b]	4-8CM	8-12CM	NL1[b]	NL2
BSQ	VIRUS	VIRUS	.50[c]*[d]	.43 *	.39 *	.36 *	.48 *	.50 *
	VIRUS	NONE	.59 *	.53 *	.27 *	.09	.0	.0
Pa405	VIRUS	VIRUS	.57 *	.52 *	.42 *	.36 *	.49 *	.49 *
	VIRUS	NONE	.62 *	.45 *	.00	.00	.00	.02

[a] INOC, leaf sections of 1st inoculation.

[b] 0-4CM, 0-4 cm from the 1st inoculated leaf section, 4-8CM, 4-8 cm from the 1st inoculated leaf section, 8-12CM, 8-12 cm from the 1st inoculated leaf section 1, NL1, 1st new leaf, NL2, 2nd new leaf.

[c] ELISA values at O.D.405.

[d] *, leaf sections contain significant amount of virus.

Data from one of two similar experiments with 8 plants per treatment.

Table 11. Spread of MDMV-B in Actinomycin D Treated Corn Plants of Varieties with Differential Resistance to the Virus as Detected by ELISA.

EXPERIMENT	VARIETY	TREATMENT	LEAF SECTION				
			INOC[a]	0-4[b]	4-8	NL1[c]	NL2
1	BSQ	AMD[d]	.50[e]*[f]	.42 *	.00	.00	.03
		WATER	.57 *	.39 *	.03	.01	.05
	MA5125	AMD	.38 *	.51 *	.56 *	.70 *	.73 *
		WATER	.66 *	.66 *	.71 *	.65 *	.84 *
2	PA405	AMD	.59 *	.05	.00	.00	.00
		WATER	.70 *	.03	.00	.00	.00
	BSQ	AMD	1.25 *	.61 *	.11	.01	.00
		WATER	.95 *	.18 *	.00	.00	.00
	MA5125	AMD	.42 *	.52 *	.54 *	.46 *	.41 *
		WATER	.45 *	.66 *	.56 *	.55 *	.84 *

[a]INOC, inoculated leaf section.

[b]cm from leaf section 1,

[c]NL1, 1st new leaf, NL2, 2nd new leaf.

[d]AMD, actinomycin D treated, water, water treated.

[e]ELISA values at O.D.405.

[f]*, sections contain significant amount of MDMV-B.

In these experiments 10 plants were used per treatment.

Table 12. Spread of MDMV-B in Actinomycin D-Treated Corn Plants of Varieties with Differential Resistance as Detected by Immunofluorescence.

	VARIETY		
TREATMENT	PA405	BSO	MA5125
ACTINOMYCIN D	6.64[a] A[b]	13.22 A	30 A
WATER	5.82 A	11.49 A	30 A

[a] distance in mm as measured by immunofluorescent technique.

[b] numbers followed by the same letter are not significantly different.

Experiment performed once with 5 plants per treatment.

Number of Infection Loci of MDMV-B in Corn

In all experiments, the numbers of infection loci continued to increase up to 36-50 hrs after inoculation and the numbers subsequently leveled off (Fig. 5). Bsq consistently exhibited more infection loci than Pa405 and Ma5125, while the numbers of infection loci in Pa405 and Ma5125 were not significantly different. Duncan's multiple range test of the data give consistent results with group 1:Bsq and group 2: Pa405 and Ma5125.

Cell-to-Cell Spread of MDMV-B

When virus was monitored by the immunofluorescent technique in all varieties virus infected cells were first detected, at 24 hr after inoculation while none could be detected at 12 and 20 hr after inoculation. At 24 hr the number of infected cells ranged from 3 (Fig. 3a) to 50. In the 1st and 2nd experiments, which were performed during winter, the rate of cell-to-cell spread was significantly higher in Bsq than in Ma5125, while it was intermediate in Pa405(Fig. 6a). Duncan's multiple range test of the data

Fig. 5. Numbers of MDMV-B infection loci in leaves of corn varieties of differential resistance mechanically inoculated with MDMV-B. a. average of 2 experiments perfo-rmed during December and January, b. one experiment perfor-med during March and April. In all experiments, the number of infection loci in Bsq was higher than that of Pa405 and Ma5125 while it was not significantly different between Pa405 and Ma5125.

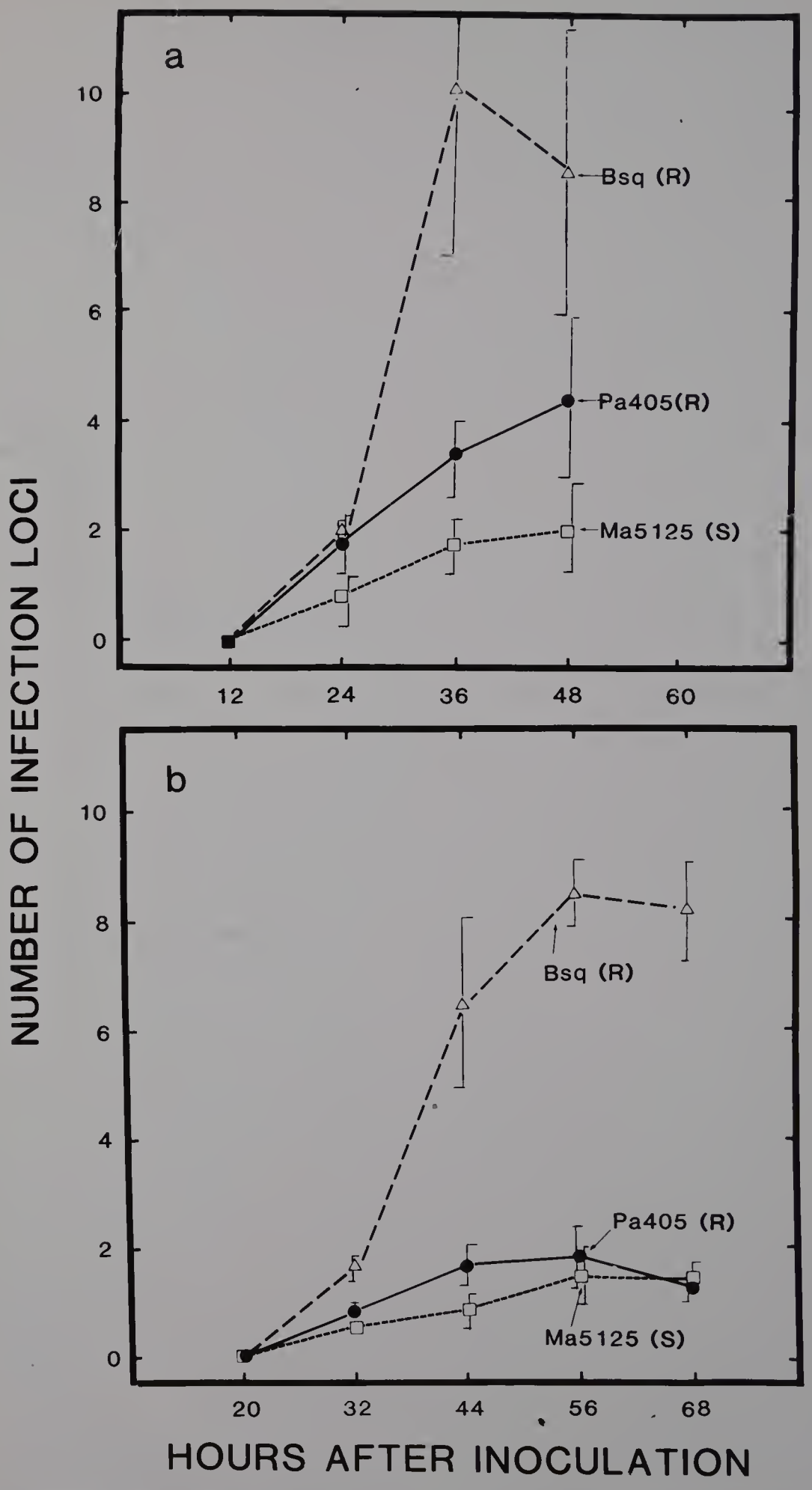
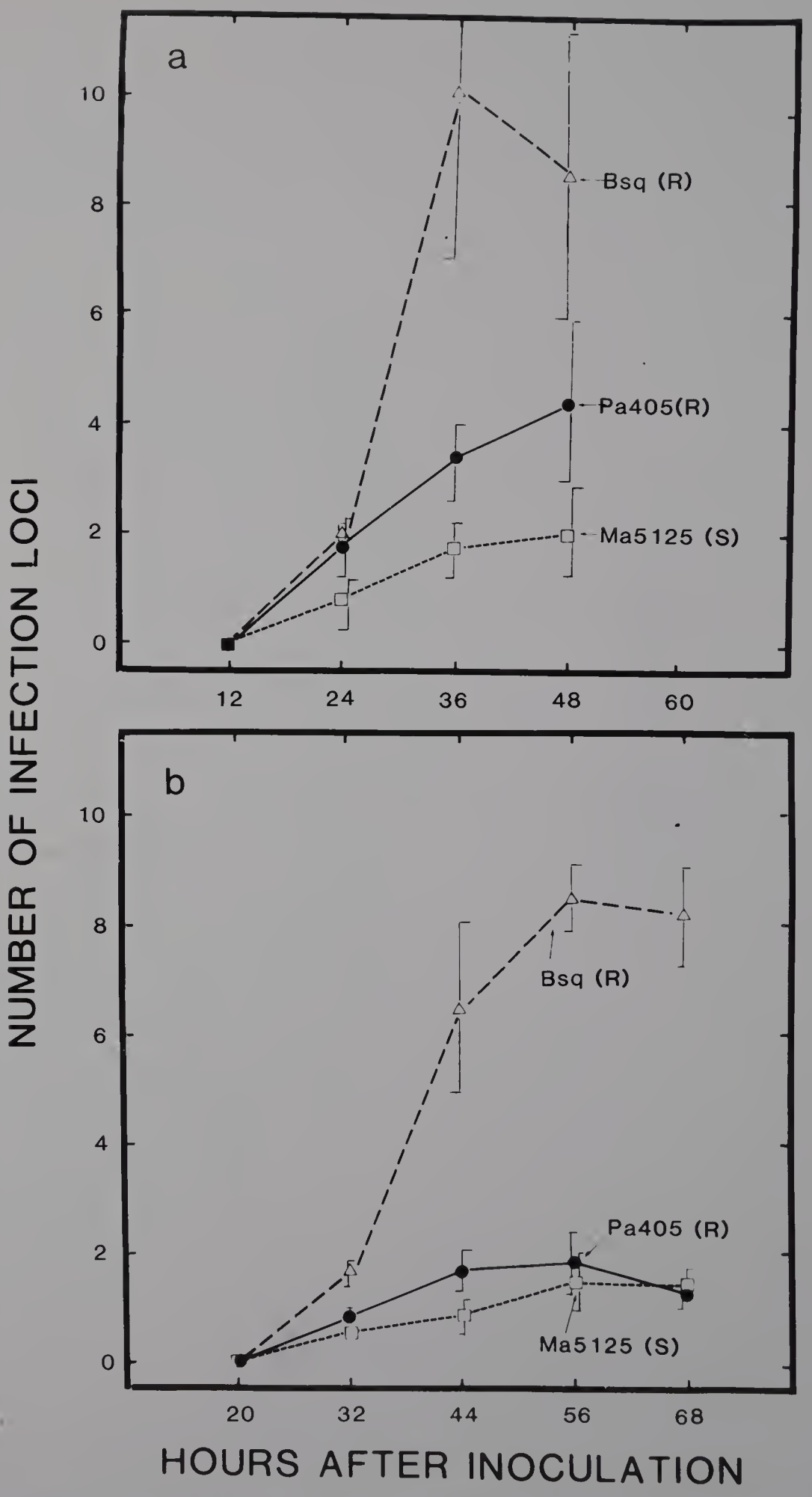

a
b
NUMBER OF INFECTION LOCI
HOURS AFTER INOCULATION
Bsq (R)
Pa405(R)
Ma5125 (S)
Pa405 (R)
Ma5125 (S)
0
2
4
6
8
10
12
24
36
48
60
20
32
44
56
68

Fig. 6. Rate of cell-to-cell spread of MDMV-B in mechanically inoculated leaves of corn varieties of differential resistance. a. average of 2 experiments performed during December and January; the rates of cell-to-cell spread of the virus were in the decending order of Bsq, Pa405, and Ma5125, b. one experiment performed during March and April; the rates of cell-to-cell spread of the three varieties were not significantly different.


a
b
Bsq (R)
Pa405(R)
Ma5125 (S)
Pa405 (R)
Ma5125 (S)
NUMBER OF INFECTION LOCI
HOURS AFTER INOCULATION
0
2
4
6
8
10
12
24
36
48
60
20
32
44
56
68

give consistent results with group 1: Bsq, Pa405 and group 2: Pa405, Ma5125. In the 3rd experiment, which was performed during spring, the rates of cell-to-cell spread in all three varieties were not significantly different (Fig 6b).

Long Tern Spread of MDMV-B

Both, resistant and susceptible varieties produced high concentrations of MDMV-B at the area of mechanical inoculation with the virus. Bsq and Ma5125 x Bsq produced significantly higher concentration of virus in sections of inoculation than the other varieties in 3 experiments performed in the summer (Table 13) but not in an experiment performed in the winter (Table 14).

When leaf sections adjacent to the inoculated areas were compared, virus concentration in Ma5125 was significantly higher than in Ma5125 x Bsq and virus concentrations in these two varieties were higher than in Pa405, Bsq, and Pa405 x Ma5125 (Table 13). Virus concentration in Pa405, Bsq, and Pa405 x Bsq were not significantly different (Table 13). This situation occurred consistently in 3 summer experiments (Table 13) but again not in the winter experiment (Table 14).

MDMV-B was consistently detected in new leaves of Ma5125 6 days after inoculation, however, the virus was

Table 13. Spread of MDMV-B in Mechanically Inoculated Leaves of Corn Varieties with Differential Resistance as Measured by ELISA.

		LEAF SECTION			
VARIETY	DAY[a]	INOC[c]	0-4CM[d]	4-8CM	NL[e]
Pa[b]	3	.17[f] AB[g]	.04 A	.00 A	.00 A
Bsq	3	.69 C	.18 B	.03 B	.02 A
Pa x Ma	3	.18 AB	.09 AB	.03 B	.06 A
Ma x Bsq	3	.24 B	.06 A	.04 B	.02 A
Ma	3	.10 A	.00 A	.00 AB	.35 B
Pa	6	.44 A	.03 A	.02 A	.02 A
Bsq	6	1.44 BC	.11 A	.07 A	.06 A
Pa x Ma	6	.73 A	.08 A	.06 A	.06 A
Ma x Bsq	6	1.57 B	.24 A	.06 A	.06 A
Ma	6	.95 A C	.74 B	.61 B	1.55 B
Pa	9	1.38 AB	.09 A	.05 A	.04 A
Bsq	9	2.60 C	.67 B	.10 A	.09 A
Pa x Bsq	9	1.16 A	.23 A	.08 A	.07 A
Ma x Bsq	9	2.57 C	2.09 BC	.83 B	.08 A
Ma	9	1.43 AB	1.51 C	1.39 B	1.62 B

[a] DAY, days after inoculation.

[b] Pa, Pa405, Ma, Ma5125.

[c] INOC, inoculated leaf section.

[d] cm from the inoculated leaf section,

[e] NL, the youngest new leaf.

[f] ELISA values at O.D.405.

[g] numbers followed by the same letter are not significantly different at each date and on each type of leaf section.

This is one of the three similar experiments performed during June-September of 1981 with 10 plants per treatment.

Table 14. Spread of MDMV-B in Mechanically Inoculated Leaves of Corn Varieties of Differential Resistance as Measured by ELISA.

VARIETY	DAYS AFTER INOCULATION	LEAF SECTION			
		INOC[b]	0-4[c]	4-8	NL[d]
Pa[a]	6	.14[e] AB[f]	.05 A	.06 A	.01 A
Bsq	6	.13 AB	.05 A	.06 A	.03 A
Pa x Ma	6	.05 B	.03 A	.04 A	.03 A
Ma x Bsq	6	.14 AB	.05 A	.04 A	.03 A
Ma	6	.15 A	.05 A	.05 A	.37 B
Pa	9	1.25 A	.21 A	.03 A	.04 A
Bsq	9	.65 B	.19 A	.03 A	.04 A
Pa x Ma	9	.16 C	.06 A	.03 A	.04 A
Ma x Bsq	9	.65 B	.08 A	.03 A	.05 A
Ma	9	.55 BC	.28 A	.04 B	1.68 B
Pa	12	1.29 A	.56 AB	.04 A	.04 A
Bsq	12	1.15 A	.25 B	.03 A	.04 A
Pa x Ma	12	.52 B	.09 B	.03 A	.05 A
Ma x Bsq	12	.45 B	.20 B	.03 A	.05 A
Ma	12	1.08 A	.74 A	.51 B	1.69 B

[a] Pa, Pa405, Ma, Ma5125.

[b] INOC, inoculated leaf section.

[c] cm from the inoculated leaf section.

[d] NL, youngest new leaf.

[e] ELISA values at O.D.405.

[f] numbers followed by the same letter are not significantly different at each date and on each type of leaf section.

Experiment was performed once during January of 1982 with 10 plants per treatment.

never found in the new leaves of the resistant varieties in these experiments. Groups of infected cells were observed on the new leaves of Ma5125 6 days after inoculation at which time spots of discoloration also began to appear (Fig. 7). Immunofluorescent staining of leaves already showing mosaic symptoms revealed that all cells, from one edge of the leaf to the other, were infected (Fig. 8).

In some experiments, MDMV-B was inoculated to 2-mm strips of leaves and then the spread of the virus into the rest of the leaf and to other leaves was monitored with the immunofluorescent technique. In Ma5125 discrete secondary infection loci, which could be traced along veins to corresponding primary infection loci in the area of inoculation, began to appear in the proximal side of the inoculation strips 6 days after inoculation (Fig. 9). No secondary loci were detected on days 2 and 4. The secondary loci were numerous and many of them fused into continuous lines of infected cells by day 9. There were few discrete secondary infection loci on the distal side of the inoculation strips. Secondary loci which could not be traced to any primary locus were observed on day 12. Most of the proximal spread of virus reached the edges of the leaf section 6 days after inoculation. A diagram of the profile of MDMV-B spread in the susceptible variety is provided for illustration (Fig. 10).

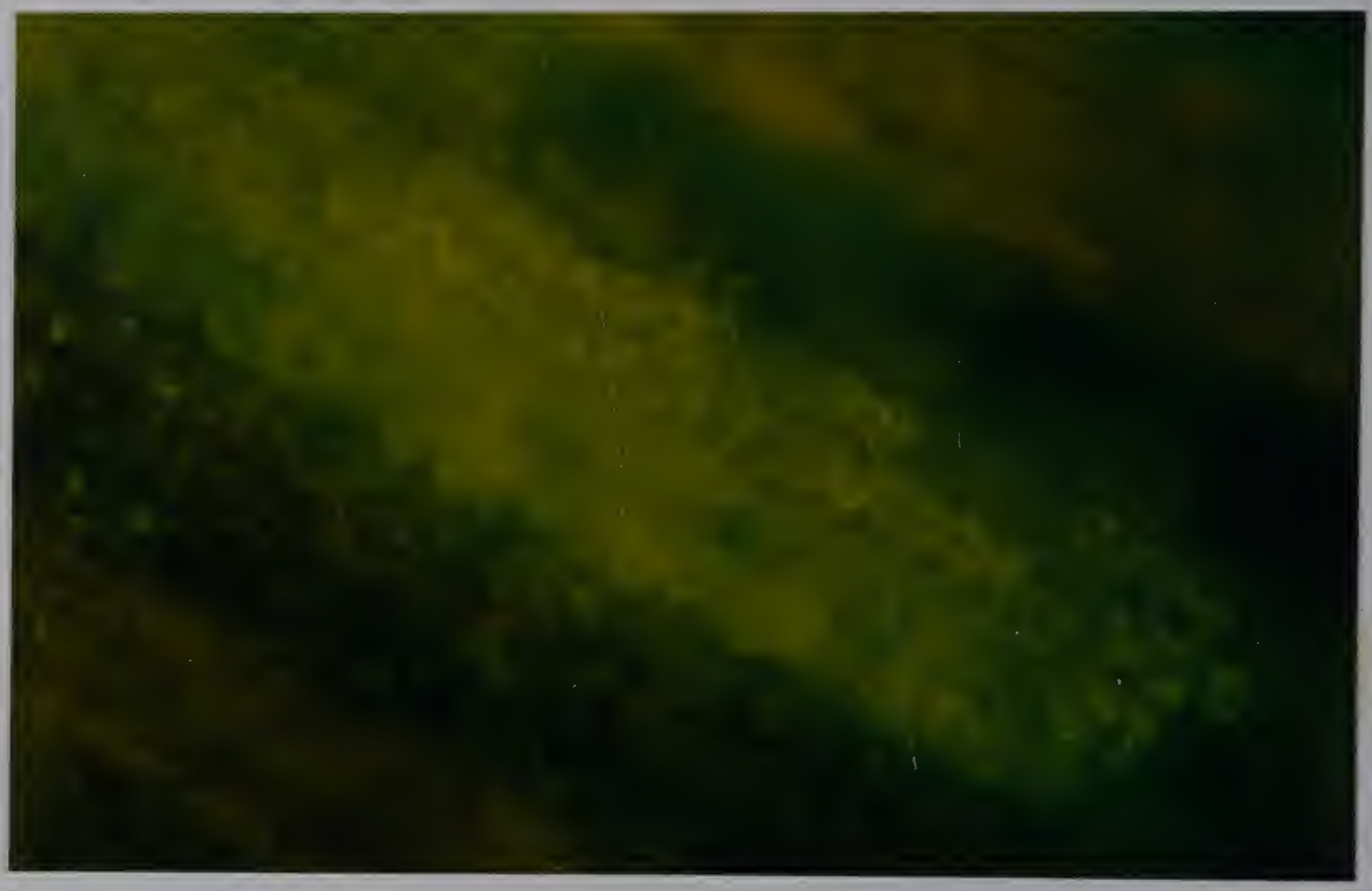


Fig. 7. MDMV-B-infected cells, in systemically infected young leaf of Ma5125 6 days after inoculation, appearing as a localized bright green fluorescent area in a dark background.

Fig. 8. Virus-infected cells giving off green fluorescence after immunofluorescent staining. Such cells covered the entire width of Ma5125 leaf showing mosaic symptoms.

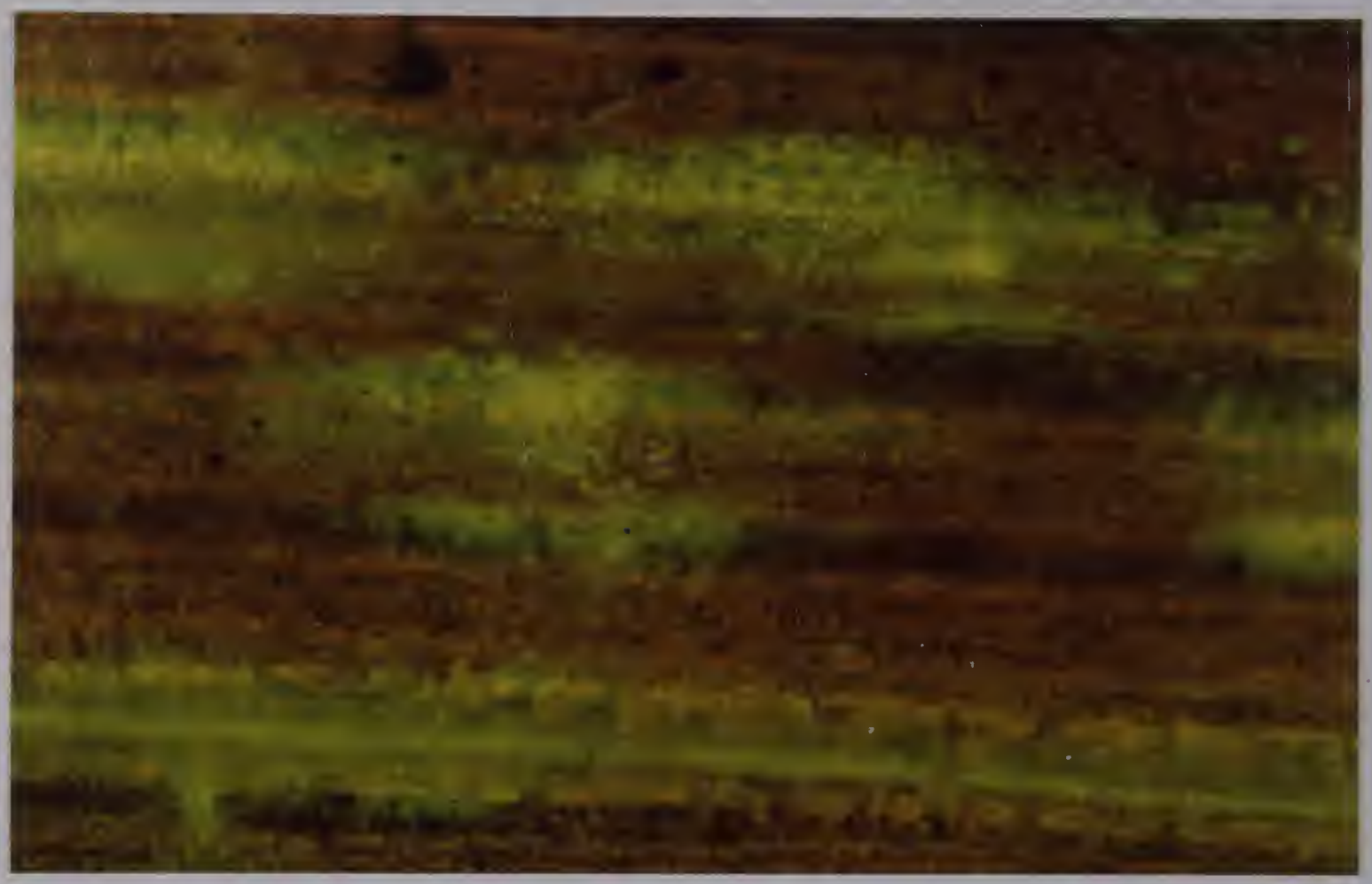


Fig. 9. Discrete secondary infection loci occurring along veins associated with primary infection loci formed at area of inoculation. In the susceptible variety Ma5125 the secondary lesions appeared 6 days after inoculation.

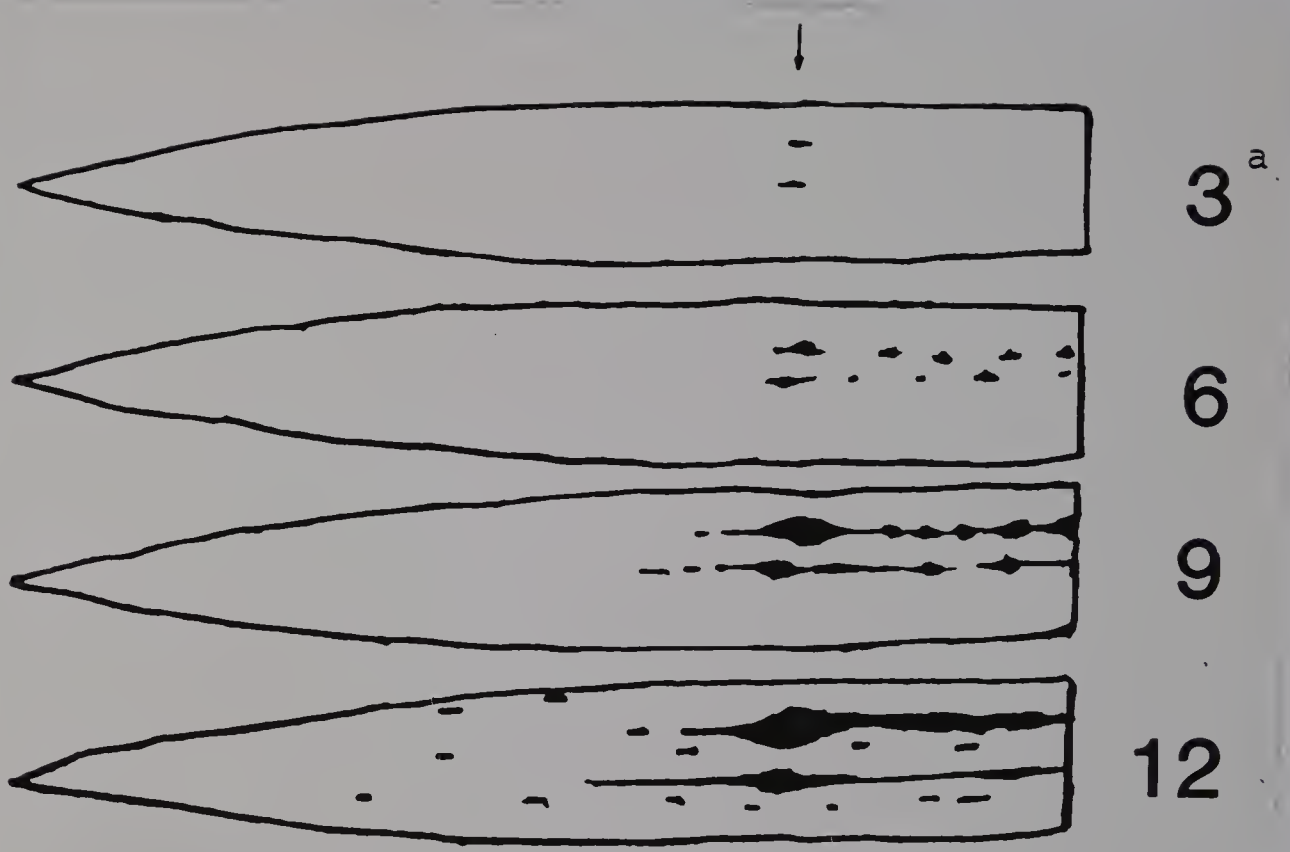
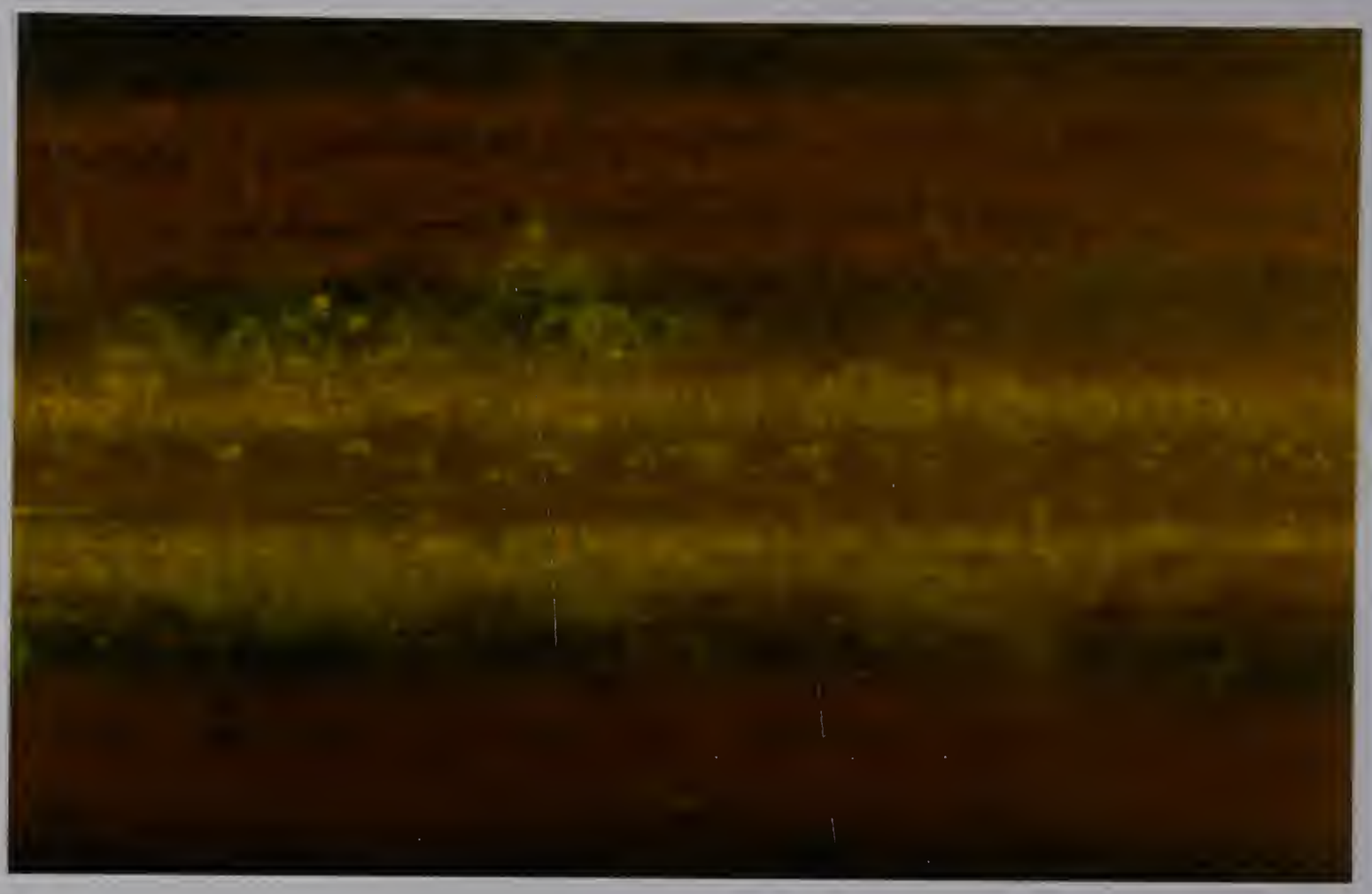


Fig. 10. Schematic representation of spread of MDMV-B in the inoculated leaves of the susceptible variety Ma5125. a. Days after inoculation. Discrete secondary infection loci occurred on the proximal side of primary infection loci (arrow) 6 days after inoculation. Such secondary loci fused into lines of continuous infected areas On day 9. Secondary infection loci which could not be traced back to any primary locus along the same vein appeared at day 12.

In Pa405 and Bsq occasional secondary loci began to appear not earlier than 9 days after inoculation. In these varieties, the spread of the virus appeared in most cases to be continuous (Fig. 11). The front edge of the virus spread is usually pointed (Fig. 12,13). A diagram of the profile of MDMV-B spread in the resistant varieties is provided (Fig. 14). The rate of long-tern spread of the virus was consistently higher in Ma5125 than in Pa405 and Bsq (Fig. 15). Duncan's multiple range test of the data from two experiments performed during November and December (Fig. 15a) gave consistent results with group 1:Ma5125, group 2:Bsq, and group 3:Pa405. Duncan's grouping of the data from one experiment performed during March and April (Fig. 15b) was group 1:Ma5125, and group 2:Bsq and Pa405.

Proximal Versus Distal Spread

When 2 mm perpendicular leaf strips were inoculated with MDMV and the distance of secondary infection loci was measured, it was shown that, in Ma5125, the distances of proximal (toward leaf base) spread of the virus were significantly longer than those of distal (toward leaf tip) spread. In contrast, the distances of virus spread in either direction were not significantly different in Pa405 and Bsq (Fig. 16).



0.1 mm

Fig. 11. MDMV-B-infected parenchyma cells of Bsq (resistant variety) stained with fluorescent antibody and appearing as columns of fluorescent cells. The position of the fluorescing cells suggests a continuous cell-to-cell spread of the virus.

Fig. 12. The front edge of an enlarging MDMV-B-infected area in a Bsq leaf showing a pointed direction of cell-to-cell spread of the virus.

Fig. 13. The pointed front edge of fluorescing parenchyma cells in an enlarging MDMV-B-infected area of a Pa405 leaf.

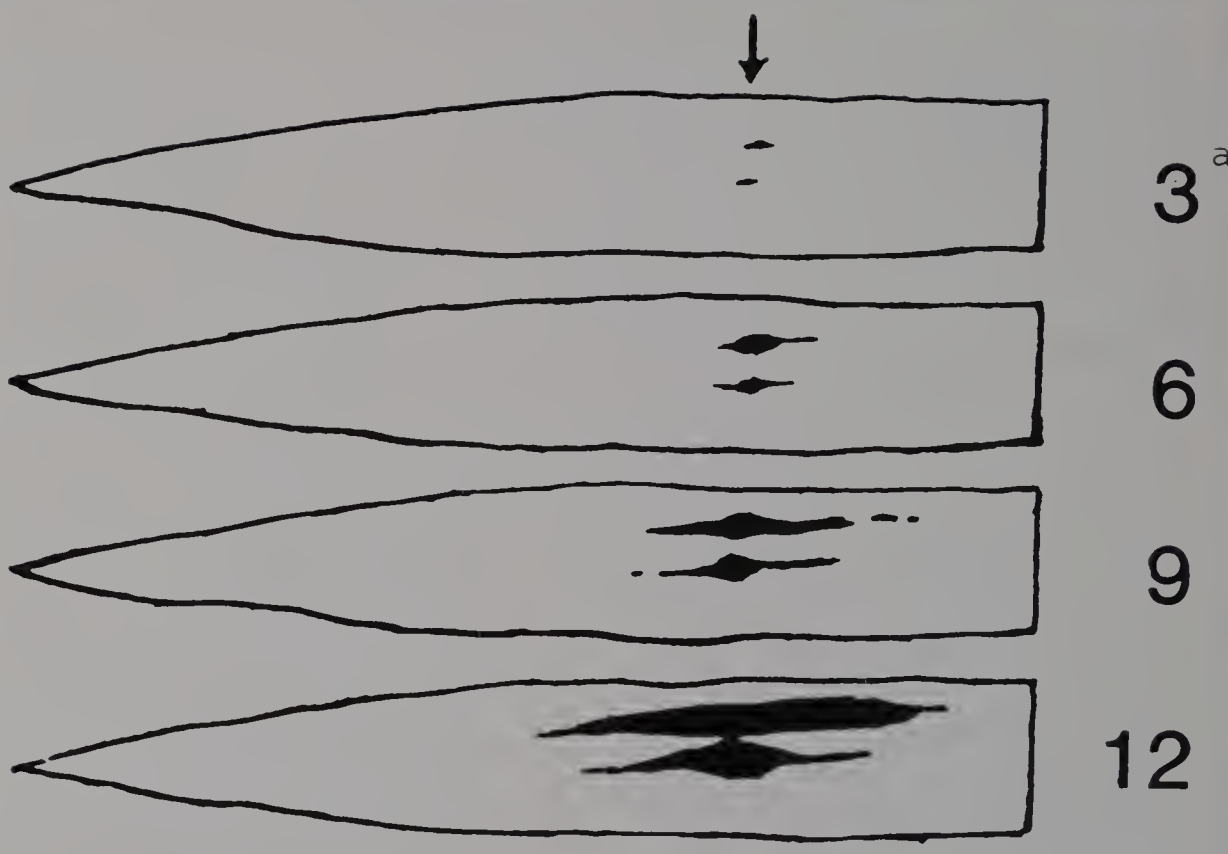
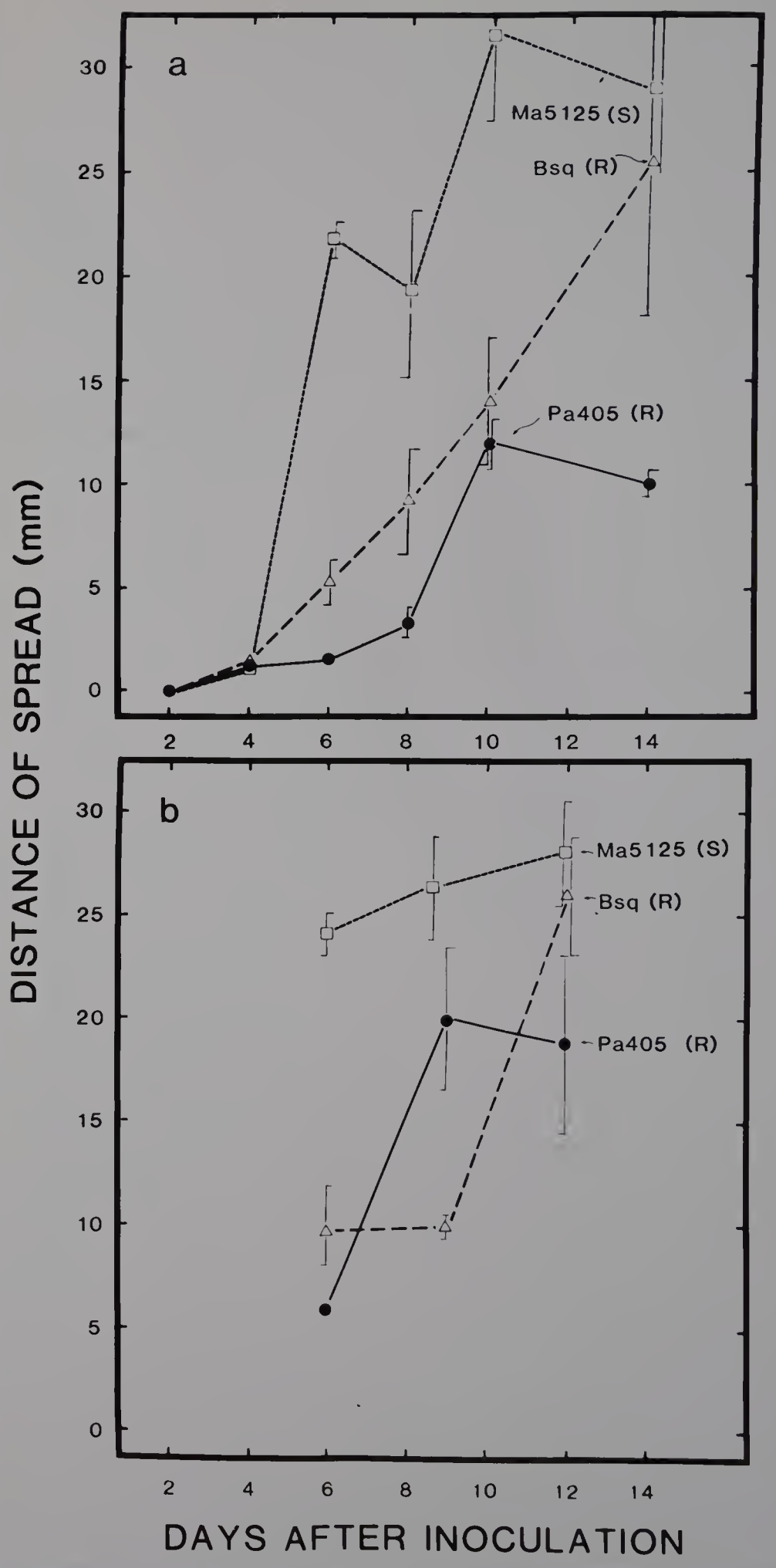


Fig. 14. Schematic representation of spread of MDMV-B in inoculated leaves of resistant varieties. a. Days after inoculation. Spread of the virus is mostly by the cell-to-cell route. Arrow indicates points of inoculation. Discrete secondary infection loci may be occassionally found 9 days after inoculation but are located near the front of a continuous cell-to-cell spread. a. Days after inoculation.

Fig. 15. Long-tern spread of MDMV-B in mechanically inoculated leaves of corn varieties of differential resistance. a. average of 2 experiments performed during November and December, b. one experiment performed during March and April. The rate of MDMV-B spread in Ma5125 was significantly higher than that in Pa405 and Bsq. The rate of virus spread in Bsq was higher than that in Pa405 during November and December (a) while it was not significantly different during March and April (b).


a
Ma5125 (S)
Bsq (R)
Pa405 (R)
b
Ma5125 (S)
Bsq (R)
Pa405 (R)
DISTANCE OF
SPREAD (mm)
0
5
10
15
20
25
30
2
4
6
8
10
12
14
DAYS AFTER INOCULATION

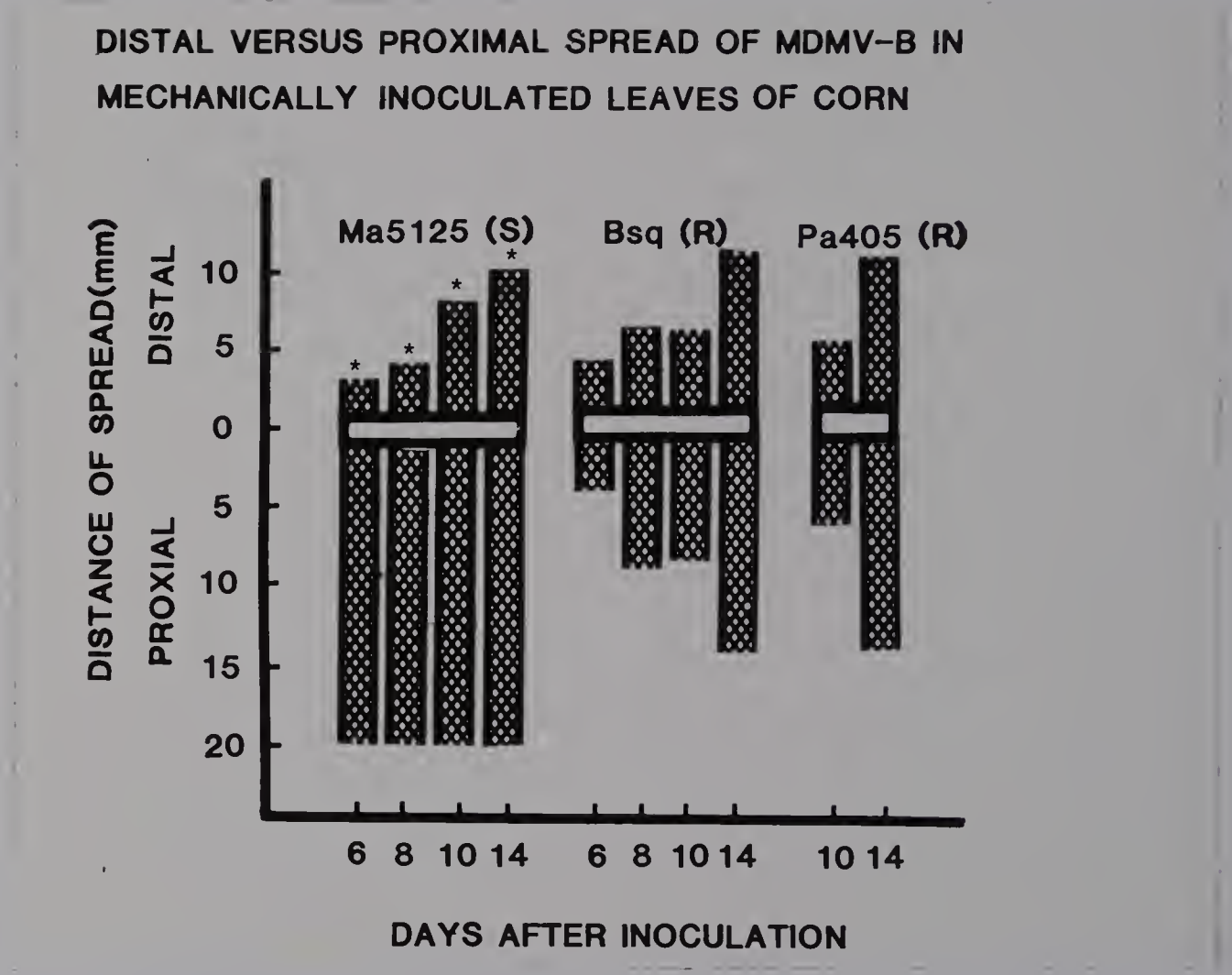


Fig. 16. Proximal and distal spread of MDMV-B in mechanically inoculated leaves of corn varieties of differential resistance. *, proximal spread significantly different from respective distal spread.

C H A P T E R V

DISCUSSION

Immunofluorescent staining of virus infected leaves partially digested by enzymes was shown to be a sensitive method that allows detailed analysis of the profile of virus spread in leaves. The method may be used as a "local lesion assay" if a local lesion-responding host for the virus is not available. This technique may also be used to study the interaction of two viruses in a plant if two different fluorochromes are used as markers of antibodies. The method has been successfully tried on corn and sorghum. It is expected that it will be possible to use this technique in studies of other host/virus interaction. The pectinase-digestion method of Nagaraj (1962) for dissociated cells and the protoplast method of Otsuki and Takebe (1969) can be used to monitor the increase in number of infected cells, but the relationship of the infected cells in the tissues can not be analyzed. The method of Hosokawa and Mori (1974) is capable of analyzing the relationship of infected epidermal cells while the method is limited to detection of infected cells in peeled epidermis. Epidermis is difficult if not impossible to peel in most monocotyledonous plants. Immunofluorescent staining of nondispersed

tissue has the advantage of uninterrupted observation of an intact large area of a leaf. Virus in epidermal cells as well as in 1-2 layers of mesophyll cells can be detected with this method.

The thirteen sweet corn inbreds that appeared resistant to MDMV-B came from the breeding program of Dr. G. Hochmuth and their genes for resistance came from the dent corn B68. None of the other sweet corn varieties in his collection were resistant. This result agrees with the report of Mikel. et al., (1984) that no resistance was found in sweet corn. Even in the resistant sweet corn inbreds, however, MDMV-B replicated and reached high titers in the inoculated leaves (Table 2).

The results of field evaluation of MDMV resistance correlated with the extent of virus spread as determined by ELISA. In these experiments (Table 13,14), Pa405, Bsq, and Pa405 x Ma5125 were highly resistant, i.e. developed no visible symptoms, and the spread of virus in inoculated leaves did not exceed 4 cm from the area of inoculation. Ma5125 x Bsq was intermediately resistant, i.e. occasionally narrow bands of chlorotic tissue would develop on leaves, and the spread of the virus reached as far as 4-8 cm from the area of inoculation. Ma5125 was always susceptible and the virus spread throughout the inoculated plants.

No immunity was found in the resistant varieties used for this work. MDMV-B replicated to high titer in the inoculated leaves of all varieties (Table 13,14) but only plants of the susceptible varieties became systemically infected. Several hypotheses can be made to explain why the virus was localized in the inoculated leaves. The hypotheses are: (1) the virus is rapidly inactivated in the resistant tissue, (2) new leaves of inoculated plants become resistant to the virus after initial replication of the virus in older leaves, (3) the virus fails to spread into or via the vascular system and, therefore, does not travel for long distance.

The first hypothesis was tested by concurrent measurement of virus concentration with ELISA and of viral infectivity with bioassay. The virus concentration was as high in the resistant varieties as it was in the susceptible ones (Table 7). The virus from resistant plants was also infectious and the infectivity correlated well with virus concentration. There was a drop of virus infectivity with time but the decrease was equivalent in both susceptible and resistant varieties. The drop of MDMV-B infectivity in susceptible varieties was also reported by Tu and Ford (1970). Thus no virus-inactivation mechanism specifically related to resistance was observed.

To test the second hypothesis the plants were first

inoculated with MDMV-B in order to elicit any potential resistance mechanism(s) and were subsequently challenge-inoculated with the same virus. The new leaves of elli-cited plants, however, did support high virus replication when they were challenged (Table 8,9). Thus, no aquired resistance was expressed by the new leaves. It was then thought that resistance may have developed but may have been localized on the inoculated leaves in the vicinity of the inoculated area and possibly just outside the front of the initial virus spread. When such areas were challenged, however,they also produced high concentration of virus (Table 10). These data suggest that no obvious transloca-table antiviral substance is produced in resistant MDMV-B-inoculated plants.

Actinomycin D inhibits DNA directed RNA synthesis. Thus treatment of plants with actinomycin D suppresses de novo synthesis of proteins at the transcription level. If the mechanism of MDMV-B resistance involves synthesis of host-coded protein after virus infection, then actinomycin D treatment should suppress or deminish the resistance of the plants and should allow wider spread of virus in the treated resistant plants. However, the extent of virus spread in treated plants was not significantly different from that in control plants (Table 12). Discrete secondary infection centers, which were a characteristic of virus

spread in the susceptible variety, were not observed in treated plants. These data suggest that no apparent active resistance mechanism at the transcription level of the plants was involved in resistance to MDMV-B. This, however, did not exclude the possibility of viral RNA acting as m-RNA in the virus-host interaction.

To test the last hypothesis the profile of spread of MDMV-B was studied with the new immunofluorescent staining technique. There was a gradual increase in the number of infection loci up to 36-50 hr after inoculation and the numbers leveled off after about 50 hr (Fig. 5). It is not exactly clear whether the increase was caused by the appearance of new detectable primary loci or by formation of secondary infection loci. Considering, however, that secondary infections in Ma5125 did not appear outside the inoculated area until 4-6 days after inoculation, it would appear that the new infection loci were mostly delayed primary loci. Leaf tissues showed heterogeneity in supporting replication and spread of the virus. The numbers of infection loci in resistant varieties were either higher or equal to those in the susceptible variety (Fig. 5). It is clear , therefore, that the resistance mechanism is not due to resistance to infection.

The rate of cell-to-cell spread of the virus was determined by measuring the dimension of the infected

areas that was parellel to the leaf veins. This approach is also based on the fact that secondary infection loci did not appear outside an inoculated area untill 4-6 days after inoculation. Thus during the first 50 hrs after inoculation virus spread via the vascular system does not contribute to the expansion of an infected area. It was found (Fig. 6) that the rates of cell-to-cell spread of the virus in resistant varieties were higher or equal to that in the susceptible variety. There is no correlation, therefore, between varietal resistance and rate of cell-to-cell spread of the virus. The resistance mechanism then could not, apprently , be due to inhibition of cell-to-cell spread of the virus.

Numerous discrete secondary infection centers, which formed along veins and could be traced back to primary infection loci, marked the more rapid spread of the virus and the appearance of systemic symptoms in the susceptible variety (Fig. 10). Such secondary infection centers were rare in the resistant varieties and appeared only near the front of the continous spread of the virus. It is not clear whether MDMV-B actually traveled via the vascular system in the resistant varieties, or the observed discrete secondary infection areas were due to spread of the virus through parenchyma cells in the deeper layers of the leaf, which were not detected by this method. The rate of spread

in the resistant varieties was lower than in the susceptible variety (Fig. 15). These data suggest that, in resistant varieties, the spread of virus via the vascular system is somehow hindered.

In the susceptible variety, proximal spread of the virus was greater than distal spread while this directional spread was not significantly different in the resistant varieties (Fig. 16). Metabolite flow in a mature leaf is mostly basipetal. The spread of virus correlated with metabolite flow in the susceptible variety but not in the resistant varieties. This phenomenon further supports the concept that the mechanism of resistance is probably through interference with virus movement into or through the vascular system.

It is not clear why MDMV-B does not move via the vascular system of resistant varieties. It would presumably be due to some kind of physical blockage of the vessels or, possibly, as suggested by Seigel, et al., (1962) some kind of recognition signal on the virus, such as viral protein coat, is involved in the vascular transport of viruses. Shalla, et al., (1982) suggested that vascular transport of virus may involve prior virus-induced modification of plasmodesmata. In that case, lack of virus ability to modify the host tissue could contribute to a resistant reaction by the host.

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